HAEMOGLOBIN FORMATION AND STORAGE IRON IN PROTEIN DEFICIENCY

BY

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Contents

Introduction 81

Chapter I. Methods 84
Determination of non-haemin iron in the tissues 84
Animals 94
Synthetic diets 95
Determination of the haemoglobin concentration 97
Red cell counts, haematocrit readings, and corpuscular constants. . 98
Determination of plasma iron 99
Determination of total haemoglobin by means of viviperfusion. . . 100
Determination of the haemoglobin remaining in the animal after viviperfusion 102
Determination of dry weight, protein and total fat of the carcase . 104
Statistical methods 105

Chapter II. Haemoglobin formation in protein deficiency. Review of the literature 106

Chapter III. Haemoglobin formation and storage iron in adult male rats on varying protein intake 113

Chapter IV a. Dietary factors in the aethiology of idiopathic hypochromic anaemia. Discussion 124

Chapter IV b. Haemoglobin formation, plasma iron, and storage iron, in growing rats on varying intake of iron and protein 127
Protein deficiency in growing rats. 129
Nutritional iron deficiency in growing rats 143
Simultaneous nutritional iron and protein deficiency in growing rats 150

Chapter V. Haemoglobin formation in pregnant and lactating rats on varying intake of protein 158
Haemoglobin formation and storage iron in the litters 171

Chapter VI. Haemoglobin formation and carcase protein in protein-depleted rats under treatment with cortisone or ACTH 173

Chapter VII. Haemoglobin formation and adrenal weight in protein-depleted rats bled by cardiac puncture 178

Chapter VIII. Quantitative studies on storage iron in man 182
The problem 182
The non-haemin iron in bone-marrow, liver, and spleen 185
The non-haemin iron in kidneys, lungs, muscles, and brain 191
The non-haemin iron in biopsy specimens of sternal marrow . . . 196

Summary 201

Acknowledgements 204

References 205

Introduction

In quantitative studies on haemoglobin synthesis much attention has been paid to the part played by iron. Since haemoglobin consists mostly of protein and only to a smaller extent of iron and porphyrin, it is surprising that so little study has been directed towards the problem of the dependence of haemoglobin formation on the supply of material for protein synthesis. To take the state of affairs in man, a person weighing 70 kg should synthesize about 8 g of haemoglobin per day, that is to say nearly 8 g of globin. Since nitrogen equilibrium can be achieved on a daily intake of 0.8 g of good quality protein per kg body-weight, a 70 kg man consuming 56 g of protein per day should be in a state of nitrogen equilibrium: in this case protein amounting to 14 % of the daily protein intake is required for haemoglobin synthesis (Drabkin, 1951).

In the presence of low protein intake some competition must exist among the various demands for material for protein synthesis. From the investigations of Whipple *et al.* (1942–1947) in particular, in which dogs were systematically bled and kept on protein-deficient diet, it has been established that haemoglobin synthesis has high priority when the supply of material for protein synthesis is inadequate. It will, however, be recalled that haemoglobin synthesis has this high priority over other body proteins under conditions where a deficit on the haemoglobin side has been artificially induced by blood-letting, and it is by no means certain that this state of affairs is general. It must be at least as important to study haemoglobin formation in relation to the synthesis of other body proteins during inadequate administration of protein to animals which have not been bled. Should new protein requirements with high priority arise—which may be presumed to include pregnancy, malignant tumours, infections with active antibody formation, etc.—it may well happen that haemoglobin formation will be affected unfavourably by the heightened competition. It would thus be of particular interest to examine the effect of low protein intake on haemoglobin synthesis in some such condition.

In the study of the degree of priority of haemoglobin formation in the competition for protein it is important to determine whether this is affected by the amount of iron available. It might be thought that abundant supplies of iron would favour haemoglobin formation by some kind of mass effect. This could possibly explain why, at least in some cases of idiopathic hypochromic anaemia, such large doses of iron are required to produce a good therapeutic effect. It has been suggested

that deficiency of protein of high biological value is a contributory aetiological factor in the development of this form of anaemia. In latent or manifest iron deficiency the mass effect of the iron would of course be insignificant, and the protein deficiency would have a more deleterious effect upon haemoglobin formation than in the presence of a surplus of iron. In any case, it would be instructive to examine haemoglobin synthesis under controlled administration of both iron and protein.

My main experiments have been devoted to haemoglobin formation under the influence of varying protein intakes in adult (Chapter III), growing (Chapter IV b), and pregnant and lactating animals (Chapter V). Pregnancy and lactation thus served as examples of conditions with heightened competition for material for protein synthesis. It is possible that experiments of this kind might be of value in helping to understand the development of certain anaemias in man.

Studies on haemoglobin formation have in the past mostly been confined to determinations of the haemoglobin concentration in the blood, little attention having been paid to the total amount of haemoglobin in the organism. To obtain a clearer, more quantitative idea of haemoglobin balance[1], however, it is essential to study the total haemoglobin. During diminished haemoglobin synthesis it may be that the fall in haemoglobin concentration is a late phenomenon, the total haemoglobin falling earlier and to a greater extent. By estimating both the total haemoglobin and the total amount of protein in the organism it is possible to get some idea of haemoglobin synthesis in relation to the synthesis of other body proteins during protein deficiency.

Previously it has been usual to draw conclusions about the iron stores on the basis of the serum iron without direct experimental support. Simultaneous estimates of the serum iron and storage iron should show clearly how close the correlation is between these two. My experiments have also served to illuminate this problem. Only by estimating the storage iron is it possible accurately to determine when the supply of iron becomes the limiting factor in haemoglobin formation. Storage iron determinations were of particular importance in the experiments in which I varied the administration of both iron and protein (Chapter IV b).

During the course of the work it became evident that the weight of the adrenals falls parallel to the total haemoglobin as the protein intake is diminished. In states of adrenal insufficiency there is a moderate fall in haemoglobin concentration, the plasma iron is low, and the total haemoglobin is reduced in relation to other body proteins: I found analogous changes in protein deficiency. It may be queried whether the fall in total haemoglobin in relation to other body proteins, which

[1] The term haemoglobin balance is used instead of haemogenic-haemolytic balance, in analogy with nitrogen balance, iron balance, etc.

occurs during protein deficiency, is not secondary to diminished adrenal activity. Animals on low protein intake were therefore treated with ACTH and cortisone (Chapter VI) in order to study the influence of these hormones upon haemoglobin and other body proteins during protein deficiency.

Since haemoglobin has priority over the proteins of the tissues and organs in haemorrhagic anaemia in animals on a low-protein diet, it should be of great interest to study adrenal function under these conditions. Low-protein animals were bled by cardiac puncture, and the weights of the adrenals recorded at the end of the experiment (Chapter VII).

Quantitative estimations of the storage iron in man under different physiological and pathological conditions are few and imperfect. Idiopathic hypochromic anaemia is commonly considered to be a pure iron-deficiency anaemia, and there is much to support this; but no quantitative, microchemical determinations of the storage iron have been carried out in this disease state. As already mentioned, deficiency of dietary protein has been suggested to be an aetiological factor. I have, therefore, in connection with my animal experiments, determined the storage iron in bone-marrow fragments obtained by sternal puncture from some cases of idiopathic hypochromic anaemia (Chapter VIII). In addition I determined post mortem the storage iron in certain organs from subjects with hypochromic anaemia.

Further, post mortem determinations of the storage iron were done in certain cases of malignant tumours and chronic infections (Chapter VIII), in which there is good reason to assume that haemoglobin synthesis is adversely affected by heightened competition for material for protein synthesis owing to tumour growth or antibody formation. In non-haemorrhagic cases of this kind one might expect an increase in storage iron. Gross *et al.* (1942) have, in fact, shown the total iron in the tissues to be high in cases of malignancy. They found it remarkable that this was so even in cases without anaemia: they had evidently not considered the possibility that the increased iron stores here too could have derived from the haemoglobin. It should, however, be possible that great changes in the total haemoglobin take place without affecting the haemoglobin concentration to any notable extent. I considered it to be of value to carry out determinations of the storage iron in cases of malignancy, paying particular attention to the intimate relationship between total haemoglobin and storage iron. The literature contains further examples of investigations on storage iron in which proper regard was not given to possible changes in the total haemoglobin. It has been shown that iron absorption is reduced in chronic infections, the storage iron, nevertheless, often being high: here again the question arises of to what extent changes in the total haemoglobin have influenced the storage iron.

CHAPTER I

Methods

Determination of Non-haemin Iron in the Tissues

Earlier Methods

The non-haemin iron in the tissues is made up, for the most part at least, of storage iron. In the quantitative determination of this iron three different types of procedure have been employed. Firstly, attempts have been made to exsanguinate the organs in question by means of perfusion, the iron then being determined after ashing (Bogniard and Whipple, 1932; Wakeham and Halenz, 1936; Austoni, Rabinovitch, and Greenberg, 1940). Determinations of this kind embrace, besides the non-haemin iron, the residual haemoglobin iron in the never wholly blood-free organ, the iron in the iron-containing enzymes, and, in the case of muscle, myoglobin iron. For organs such as bone-marrow and the spleen this method is hardly practicable, since it is impossible to get them anything like exsanguinated. Secondly, the haemoglobin has been extracted from the tissues as haematin: the non-haemin iron can then be estimated as the difference between the total iron and the haematin iron (Schaefer, 1940), or the residual iron can be determined directly (Scott, 1945). Thirdly, the method of extracting the non-haemin iron from the tissues has also been employed: the extraction medium used for this purpose has either been acid—most commonly hydrochloric acid—of varying strength, or else a buffer solution with or without the addition of reducing substances. Reduction is of importance because the ferrous ion has not the same tendency as the ferric to form complexes with proteins. Sodium pyrophosphate is another substance which is often used to free the ferric ion from its attachment to protein.

Starkenstein and Weden (1928) appear to have been the first to use acid in the extraction of non-haemin iron. They boiled tissue fragments in 5 N hydrochloric acid, and report that the haemoglobin iron was not split off in measurable quantity. McFarlane (1932) has since employed this method. Sherman, Elvehjem, and Hart (1934) also boiled the homogenized tissue sample in 5 N hydrochloric acid during 10 minutes. Borgen and Elvehjem (1937) homogenized 3–5 g of liver in 5 ml of 10 % trichloroacetic acid, and heated the suspension in a water-bath at 85–90° C during 5 minutes. Andersson (1950)

hydrolysed a tissue suspension in dilute hydrochloric acid (0.67 %) at 37° C during 48 hours in order to extract the storage iron: he maintains that the use of stronger acid would not have increased the yield; neither was it influenced by the addition of reducing substances. Nylander (1953) homogenized the tissue mixed with water in an amount corresponding to 10 times the weight of the tissue: 1 ml of this suspension was mixed with 1 ml of 0 N hydrochloric acid and hydrolysed during 48 hours at 37° C under continuous shaking.

Extraction is incomplete when buffer solutions or very dilute acid are employed. Sherman, Elvehjem, and Hart (1934) found that in the case of certain organs the use of buffer resulted in the extraction of only a small part of the non-haemin iron. By increasing the degree of acidity (4 % or 10 % acetic acid was used) they were able approximately to treble the yield of non-haemin iron from liver. They therefore abandoned a buffer extraction method which they had worked out themselves (Elvehjem, Hart, and Sherman, 1933); in this the sample was suspended in 5 ml of sodium acetate-acetic acid buffer of pH 5, about 0.25 g of sodium hydrosulphite being added to reduce the iron to the ferrous form. Shackleton and McCance (1936) described a buffer extraction method differing from that of Elvehjem *et al.* chiefly in that the suspension was heated to 100° C during 10 minutes. Concerning the use of reducing media, suffice it to say that Borgen and Elvehjem (1937) have shown that thioglycolic acid releases iron from haematin, whereas sodium hydrosulphite ($Na_2S_2O_4$) and cysteine do not. Shorland and Wall (1936) have demonstrated that thioacetic acid releases iron from haemoglobin.

Tompsett published in 1935 a method in which he used a solution of sodium pyrophosphate for extracting the non-haemin iron. He ground 10 g of finely divided tissue in 20 ml of a 4 % solution of sodium pyrophosphate, and allowed the mixture to stand for 15 minutes before precipitating the proteins. Tompsett had previously shown (1934) that when the proteins in a suspension of egg or egg-yolk are precipitated with trichloroacetic acid, no iron is found in the filtrate; nor is iron present in the filtrate after the addition of inorganic iron to the egg. By treating the suspension of egg-yolk with thioacetic acid, sodium pyrophosphate, or sodium hydrosulphite, before precipitation with trichloroacetic acid, Tompsett recovered the same amounts of iron as after ashing. Borgen and Elvehjem (1937) have shown that the addition of sodium pyrophosphate results in low iron values, owing to partial precipitation of the iron as ferric pyrophosphate. By first adding thioglycolic acid they succeeded in preventing the precipitation of ferric pyrophosphate—their explanation of this was that the iron formed a compound with the thioglycolic acid. Brückmann and Zondek described in 1940 their "hot pyrophosphate" method of extracting the non-haemin iron: 1 g of finely divided tissue is heated in a mixture of 5 ml of a

saturated solution of sodium pyrophosphate and 10 ml of trichloroacetic acid in a boiling water-bath for 7 minutes. They maintain that by this method all non-haemin iron is extracted, and that yields are obtained which closely correspond to those resulting from indirect determinations (total iron–haemoglobin iron). Brückmann and Zondek point out that Tompsett's extraction-time was too short for complete extraction with sodium pyrophosphate. If extraction is to be total at room-temperature it must proceed for at least 24 hours; if on the other hand the sample is heated, all non-haemin iron is extracted in a few minutes. Brückmann and Zondek believe that with their method there is no risk of the iron being precipitated as ferric pyrophosphate, and that under the conditions of their method a very stable, complex, iron-pyrophosphate anion of the type $[Fe_2(P_2O_7)_3]------$, which is soluble in water and acids, would be rapidly formed. The mechanism of extraction with sodium pyrophosphate does not, however, seem to be quite clear.

The Method Used in this Investigation

Of the three methods for determining the non-haemin iron described above the direct-extraction procedure, with acid as extraction medium, seems the most likely to give reliable results. The particulars of this method to be found in the literature, however, are such as to make thorough experimental trials necessary. The requirements of an extraction method of this kind are, firstly, that the haemin iron is not liberated, and, secondly, that all non-haemin iron is indeed extracted. To determine the greatest strength of acid which can be used, and for how long the extraction can be continued at, say, 95° C without liberating more than negligible amounts of iron from the haemoglobin by hydrolysis, crystallized carboxyhaemoglobin was subjected to hydrolysis with hydrochloric acid of different concentrations and for various periods of time at the temperature in question.

The carboxyhaemoglobin was prepared from horse blood in the following manner. Defibrinated blood was saturated with pure carbon monoxide, the serum separated off by centrifuging, and the blood corpuscles washed 4 times in an equal volume of 1.5 % sodium chloride. For every 100 ml of packed cells 30 ml of peroxide-free ether was added under continuous shaking; haemolysis then occurred and the carboxyhaemoglobin crystallized out. On centrifuging, the cell mass accumulated in a cake over the crystalline carboxyhaemoglobin. The carboxyhaemoglobin was then dissolved in 1.5 % sodium chloride, and the solution dialysed against running tap-water during 72 hours, and against distilled water for a further 48 hours. That part of the carboxyhaemoglobin which then crystallized out was separated off by means of centrifuging, and the crystalline carboxyhaemoglobin dissolved in 1.25 % sodium chloride. 1 ml of this solution contained 0.197 g of carboxyhaemoglobin.

One ml of the above carboxyhaemoglobin solution was added drop by drop to 10 ml of hydrochloric acid varying in strength from 1 to 6

Table 1. Iron split off from crystallized CO-haemoglobin by hydrolysis with hydrochloric acid of different concentrations. Temperature 95° C. Time 6 hours.

HCl normality	Iron split off, µg per 0.197 g CO-haemoglobin	Iron split off %
1	—	0
2	—	0
3	—	0
4	5.3	0.8
5	14.2	2.1
6	15.8	2.4

normal, and the mixture hydrolysed in stoppered flasks at 95° C during 6 hours. After cooling, the proteins were precipitated with trichloroacetic acid, the sample centrifuged, and the iron determined with *o*-phenanthroline as described below (see p. 93). The results are summarized in Table 1. No iron is released from the carboxyhaemoglobin until the concentration of the acid has reached 4 normal. On hydrolysis with 4 N HCl for 6 hours at 95° C about 0.8 % of the haemoglobin iron is split off; with 5 normal and 6 normal acid about 2 % is split off.

One ml of the carboxyhaemoglobin solution was then hydrolysed in 10 ml of 5 N HCl at 95° C for different periods of time varying between 15 minutes and 8 hours. The results are given in Fig. 1: it can be seen that 1–2 % of the haemoglobin iron is more easily split off than the rest. The carboxyhaemoglobin remaining after this fraction of the haemoglobin iron has been split off is more stable. The same amount

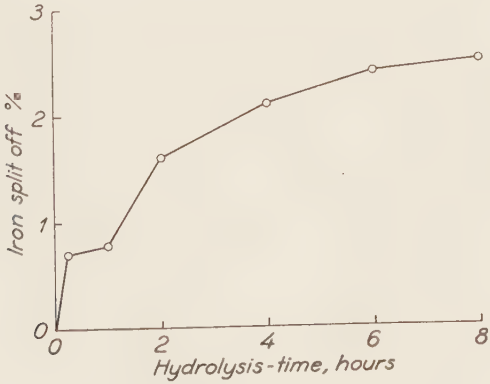

Fig. 1. Effect of varying hydrolysis-time on the amounts of iron split off from crystallized CO-haemoglobin on hydrolysis with 5 N hydrochloric acid. Temperature 95°C.

of iron is liberated from the haemoglobin in 15 minutes at 18° C as in 15 minutes at 95° C. Porphyrin fluorescence is readily demonstrable after 2 hours at 95° C.

Barkan (1927–1939) has shown that a certain fraction of the iron in the red blood corpuscles is liberated by dilute hydrochloric acid. This he called "easily split-off" iron. He hydrolysed blood, diluted 1: 10 with distilled water, in an equal volume of 0.8 % hydrochloric acid at 37° C. In less than 24 hours a yield of ionized iron was obtained which was not increased by continuing the hydrolysis for days or even weeks. Using ox blood he found that 17 mg of iron was liberated per litre. The easily split-off iron was independent of the haemoglobin concentration of the blood. It was split off without porphyrin being demonstrable, which led Barkan to suppose that it originated from a bile pigment haemoglobin in the erythrocytes. The presence of carbon monoxide or the reduction of the oxyhaemoglobin to haemoglobin prevented the liberation of two-thirds of the easily split-off iron. Legge and Lemberg (1941) have confirmed Barkan's findings. They are of the opinion, however, that only that third of the easily split-off iron which is liberated in the presence of carbon monoxide comes from a bile pigment haemoglobin in the red cells, the remaining two-thirds being formed from oxyhaemoglobin by the effect of the acid. On acidification the oxygen in the oxyhaemoglobin becomes strongly active and able to oxidize parts of the haemoglobin molecule. Using radioactive iron, Grinstein and Moore (1948) seem to have proved that easily split-off iron is an "artefact" which originates entirely or almost entirely from intact haemoglobin. Several researchers (Burmester, 1937; Venndt, 1940; Barkan and Schales, 1942) have shown that the quantity of iron detached depends upon the concentration of the acid used: less iron is liberated in strong acid than in weak. Venndt demonstrated that hydrolysis of human blood in 2 % hydrochloric acid for 1 hour at 25° C resulted in about 7 µg ionized iron per ml of blood, hydrolysis in 0.4 % hydrochloric acid for the same length of time yielding 12 µg iron. After 24 hours the corresponding values for 2 % and 0.4 % hydrochloric acid were 12 µg and 18–19 µg of ionized iron per ml blood respectively. Starkenstein and Weden (1928) boiled blood from five patients in 5 N hydrochloric acid, and obtained 15–22 µg of ionized iron per ml blood.

Since I used 5 N HCl to extract the non-haemin iron from the tissues I have examined the effect of 5 N HCl on the blood of rats and man. One ml of blood was hydrolysed in 10 ml 5 N HCl at various temperatures between 40° C and 110° C for different lengths of time. Fig. 2 shows the effect of temperature on the amount of iron detached from rat blood after hydrolysis for 1 hour. Each mark on the curve represents the mean of 5–10 determinations on rat blood with a haemoglobin level of 14.5–16.5 g per 100 ml. At about 85° C the amount of iron detached begins to increase more sharply as the temperature rises.

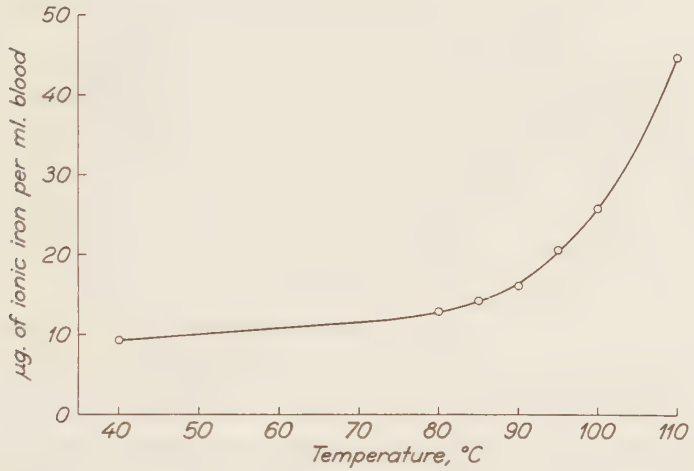

Fig. 2. Effect of temperature on the amount of iron split off from rat blood by 5 N hydrochloric acid. Hydrolysis-time 1 hour.

In order to find out how rapidly the splitting off of the iron proceeds, rat blood was hydrolysed in 5 N HCl for different lengths of time from 15 minutes to 3 hours. The curves in Fig. 3 demonstrate how the quantity of detached iron increases with time at temperatures of 95° C and 100° C respectively. Weak porphyrin fluorescence could be demonstrated in the hydrolysate after 45 minutes. It can be seen from the

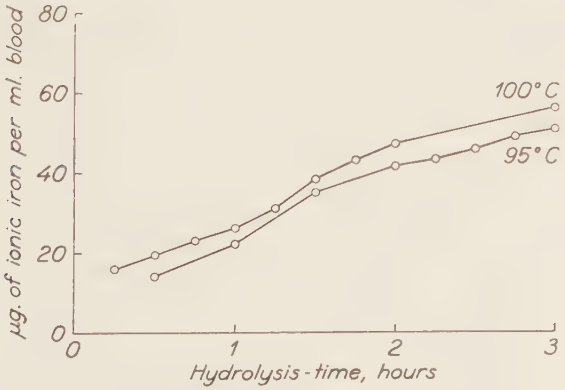

Fig. 3. Effect of varying hydrolysis-time on the amount of iron split off from rat blood on hydrolysis with 5 N hydrochloric acid. Temperature 95 or 100°C.

curves in Figs. 2 and 3 that some of the iron in the erythrocytes is more easily split off than the rest.

The effect of 5 N HCl on human blood was not investigated systematically. Hydrolysis in 5 N HCl for 1 hour at 95° C of 1 ml of blood from each of 5 healthy blood-donors, however, gave somewhat lower yields of detached iron than those obtained for rat blood. To extract the non-haemin iron from an organ, I hydrolysed the tissue with 5 N HCl at 85–90° C for 1 hour. Under these conditions about 16 μg of iron is split off from 1 ml of rat blood. Scott and McCoy (1944) give the following figures for the blood content of different organs in rats: liver 8 ml, spleen 30–35 ml, bone-marrow 12 ml, and muscle 2 ml per 100 g tissue. (The blood content in the organs was calculated from the difference between total iron and non-haemin iron.) Thus, of the hydrolysable iron in 1 g of rat liver containing blood about $\frac{8 \times 16}{100} =$ $= 1.3$ μg iron should come from the blood in the tissue, and in 1 g of splenic tissue about $\frac{30 \times 16}{100} = 4.8$ μg of the hydrolysable iron should originate from the blood. Even when the organs contain no storage iron, this iron liberated from the haemoglobin constitutes only a small fraction of the hydrolysable iron in the tissues. In exsanguinated rat liver containing no storage iron I found about 10 μg of hydrolysable iron per g of tissue. Spleen from iron-depleted rats perfused in vivo contained 20–30 μg of hydrolysable iron per g of tissue. Lewis, Goodman, and Shuck (1950) obtained higher figures for the blood content of individual organs in rats than those given above. They determined the plasma volume by extracting T-1824 (Evans' blue dye) from the tissues, and the red cell mass from the haematin content of the same tissues. According to Lewis *et al.* the liver contains 0.178 ± 0.012 ml of blood per g of tissue, viz. about twice the quantity reported by Scott and McCoy. The argument outlined above holds even in the light of these figures.

In the animal experiments of this investigation the non-haemin iron of viviperfused rats was determined: here only in bone-marrow and spleen has the presence of blood to be taken into consideration. Storage iron was also determined in human autopsy material, in which, of course, all organs contain blood. In determining non-haemin iron in muscle no iron is released from the myoglobin: myoglobin revealed itself to be very stable on treatment with acid, no free iron being demonstrable after hydrolysis of pure myoglobin in 5 N HCl for 1 hour at 95° C.

Summing up the results of the first part of the method-testing it is possible to say that the method of extracting the non-haemin iron from the tissues with 5 N HCl for 1 hour at 85–90° C fulfils the condition of not liberating more than insignificant quantities of haemin iron.

Table 2. Effect of varying extraction-time on the amounts of iron extracted from pig liver with 5 N HCl. Temperature 85–90° C.

Extraction-time	µg iron extracted per g pig liver
15 min.	Opalescent solution
1 hour	346
2 hours	350
4 hours	359
6 hours	365
8 hours	363

The second part of the method-testing was designed to establish (1) the strength of acid required, and (2) the extraction-time required for extracting the non-haemin iron as completely as possible. Approximately 1 g of homogenized (Turmix blender) pig liver was hydrolysed in 10 ml 5 N HCl at 85–90° C for periods varying from 15 minutes to 8 hours (see Table 2). After extraction for 15 minutes the solutions were opalescent, and remained so even after precipitation of the proteins. (This opalescense was probably due to glycogen which had still not been broken down to glucose.) After extraction for 1 hour the solutions were clear. If extraction was continued for longer than 1 hour slightly more iron was obtained, an increase no greater than can be explained by further splitting off of iron from the haemoglobin with prolonged extraction-time. After extraction of the iron the protein was precipitated with 20 % trichloroacetic acid, the specimens centrifuged, and the clear

Table 3. Comparison of the amounts of iron extracted from pig liver with 4 N HCl and 5 N HCl. Temperature 85–90° C. Time 1 hour.

µg iron extracted per g liver	
4 N HCl	5 N HCl
329	355
329	355
319	347
318	346
318	348
331	359
$m_1 = 324$	$m_2 = 352$

$d = m_2 - m_1 = 28$

$P < 0.001$.

Table 4. Recovery experiments.

µg iron in the sample of pig liver	µg iron (as Mohr's salt) added to the liver	µg iron recovered	% recovery
565.6	43.2	39.4	91
286.9	86.3	79.5	92
251.6	86.3	95.0	110
291.0	86.3	88.3	102
412.1	172.7	178.7	103
495.2	172.7	169.2	98
344.4	172.7	170.2	99
359.9	172.7	181.9	105
221.9	86.3	87.7	102
267.0	86.3	92.6	107
161.4	172.7	165.0	96

supernatant decanted. The precipitate was washed twice in a mixture of equal parts of 5 N HCl and 20 % trichloroacetic acid: only traces of iron could be extracted from this precipitate on repeated hydrolysis with 5 N HCl for 1 hour. I also attempted to extract the non-haemin iron using acids weaker than 5 N HCl. Hydrolysis with weaker hydrochloric acid for 1 hour at 90° C did not result in total extraction. The solutions were often not clear, and the tissue not so finely particulated as with 5 N HCl. It was sometimes possible to obtain clear solutions by filtration, but this always involved the loss of some of the iron on the filter-paper. Even 4 N HCl did not give such complete extraction as 5 N HCl (see Table 3): this was probably because the 5 N acid breaks down the tissue more effectively. Trials at lower temperatures also gave unsatisfactory results.

Further tests of the extraction using 5 N HCl were done by adding known quantities of iron salt to weighed amounts of homogenized pig liver, the non-haemin iron content of which had been previously determined by means of a large number of analyses. In 11 experiments 91–110 % recovery was obtained (Table 4).

The second section of the method-testing thus proved it possible to extract all the non-haemin iron by hydrolysing the tissue with 5 N HCl for 1 hour at 85–90° C. A detailed description follows of the method I have worked out for determining the non-haemin iron in tissue.

Procedure

Parenchymatous organs such as liver and spleen were homogenized in a porcelain mortar by means of two glass knives. Rat carcases were finely minced in a Turmix blender the blades of which were coated

with a thin layer of silicone grease: repeated controls showed that the homogenized tissue was not contaminated by iron from the apparatus.

Approximately 1 g of homogenized tissue was weighed into a stoppered weighing-bottle. The specimen was hydrolysed in 10 ml of 5 N HCl in a stoppered flask for 1 hour in a thermostat at 85–90° C. After cooling, the specimen was decanted into a centrifuge tube, and 10 ml of 20 % trichloroacetic acid added to precipitate the proteins. The specimen was allowed to stand for at least 15 minutes, after which it was centrifuged. The clear, yellowish-brown supernatant was decanted into a conical flask. The precipitate was washed twice in a mixture of equal parts of hydrochloric and trichloroacetic acid. Two drops of bromocresol purple were added to the acid-extract, and the solution neutralized with concentrated ammonia to colour change. The solution was retitrated with 0.5 N H_2SO_4 to new colour change, and a further 3 ml of 0.5 N H_2SO_4 added. The solution was made up to 100 ml with distilled water; it was now pale yellow with a pH of 2.2–2.4. This is a suitable degree of acidity for determining iron by the *o*-phenanthroline method. Next 0.5 ml of 2 % hydroquinone and 0.5 ml of 1 % *o*-phenanthroline hydrochloride were pipetted into a 25 ml measuring flask, and the flask filled up to the mark with the dilute extract. After 12 hours the extinctions were read off in a Beckman spectrophotometer, model DU, at 500 mμ. To correct for the pale yellow colour of the solution each specimen was read against a blank in which the *o*-phenanthroline hydrochloride had been replaced by a corresponding amount of water. Another blank was read to correct for the iron contained in the acids and other chemicals used. Ferrous ammonium sulphate (Mohr's salt), which was shown to be free from ferric iron on testing with potassium thiocyanide, was used as iron standard. The iron standard was titrated with potassium permanganate.

Trials with different quantities of *o*-phenanthroline hydrochloride showed that at least 35 μg of this reagent is required for every μg of iron if the colour is allowed to develop for 12 hours. The quantities of iron present in the samples were usually such that the reagents were used in the amounts mentioned.

The standard errors of the iron determinations in certain tissues with varying iron content are given in Table 5. Double determinations have been performed throughout.

Some comments on the method used in this work

The determinations by this method of non-haemin iron in blood-containing tissues embrace, in addition, a fraction of haemin iron ("easily split-off" iron) from the red blood cells. It is probably impossible to extract all non-haemin iron from a tissue without at the

Table 5. Standard errors of non-haemin iron determinations in various tissues.

Material	Hydrolysable iron, mg per 100 g fresh tissue	*N*	Standard error of a single determination	Standard error of the mean of a double determination
Pig liver	25.0 –36.0	20	0.72	0.51
Rat liver	10.0 –20.0	21	0.26	0.18
,, ,,	6.0 –10.0	20	0.26	0.18
,, ,,	3.0 – 6.0	22	0.20	0.14
,, ,,	1.0 – 3.0	25	0.14	0.10
,, ,,	0.5 – 1.0	29	0.08	0.06
Rat carcase	1.5 – 3.0	29	0.068	0.048
,, ,,	1.0 – 1.5	45	0.050	0.035
,, ,,	0.60– 1.0	34	0.046	0.033
,, ,,	0.30– 0.60	38	0.036	0.025

N = Number of double determinations.

same time removing this fraction from the blood, but the quantity of easily split-off iron is insignificant in comparison with the non-haemin iron. On heating to 100° C in 5 N HCl some of the more stably bound haemoglobin iron is split off. This can be almost completely avoided by carrying out the hydrolysis at a slightly lower temperature, viz. 85–90° C. By hydrolysing the tissue in 5 N HCl for 1 hour at this temperature it is reduced to a finely divided state, which expedites extraction. This method gives total extraction of the non-haemin iron, and there is no risk of the iron being precipitated as insoluble salt. A great advantage is that the tissue is not mixed with water for homogenizing. Finally, the error of the method is comparatively small even when the tissue contains little storage iron.

Animals

Rats were chosen for this investigation. They are particularly suitable for determination of the total haemoglobin by means of viviperfusion, and for estimation of the total protein in the carcase. White, inbred Wistar rats were used. The stock animals were obtained from the National Institute for Public Health, Stockholm. These animals are free from parasites and infection[1], and are excellent for studies on metabolism.

The stock animals were fed on a diet of the following composition: powdered whole milk 600 g, whole-meal wheat flour 880 g, wheat germ 300 g, dried yeast 100 g, poppy seed oil 100 g, calcium carbonate 10 g,

[1] Splenectomy showed the animals to be free from haemobartonella infection.

and sodium chloride 10 g (total 2 000 g). For every 2 000 g of this mixture were added 60 g of cod-liver oil (which amounts to 3 % of the above), 1.6 g of ferric citrate and the following quantities of four salt-solutions, each containing 1 g of salt per litre: 12.5 ml NaF; 9.0 ml $KAl(SO_4)_2 \cdot 12\ H_2O$; 25 ml $MnSO_4 \cdot H_2O$; and 5 ml KI. The dietary ingredients were mixed thoroughly in a Turmix. The animals were given this food *ad lib.*, water being admixed to prevent scattering.

This stock diet has been in use for many years at the Vitamin Department of the Swedish National Institute for Public Health. On it Wistar rats have a very high fertility.

During the experiments the animals were kept in individual cages of chromium-plated brass netting. This material was used to eliminate a source of iron contamination when the animals nibbled the cages. The meshes were wide enough to prevent coprophagia, but in the case of pregnant and lactating animals, the cages were placed on aluminium sheeting.

Synthetic Diets

In the past, milk diets have been used for inducing nutritional iron-deficiency anaemia in growing rats (Elvehjem and Kemmerer, 1931), but these diets also produce a complicating copper deficiency. No synthetic diet which produces iron-deficiency anaemia is described in the literature. In order to be able to study the formation of haemoglobin on varying intake of iron and protein it was necessary to compose a synthetic diet poor in iron. In this connection it was very difficult to find a protein with extremely low iron content. Spray-dried hen's egg-white, however, contains very little iron, viz. 4–7 µg of iron per gram, and this was used. The avidin in it was denatured by boiling the protein in absolute alcohol, which was then evaporated. As further suitable ingredients in a synthetic iron-deficiency diet were chosen saccharose, poppy-seed oil, an iron-free mixture of salts, and adequate quantities of vitamins. The composition of the salt mixture was that given in the Swedish Pharmacopoeia for the biological standardization of vitamins A and B_1, the iron salt excluded. A synthetic diet containing, say, 18 % protein had the following composition:

Spray-dried hen's egg-white (Källbergs Industri, Ltd., Töreboda, Sweden)	18 %
Saccharose	73 %
Poppy-seed oil (Karlshamn's Oil Works)	5 %
Salt mixture (iron-free)	4 %

As the protein of the diet was cut down it was replaced by a corresponding amount of saccharose. For every 100 g of this diet were added 5 µg

Table 6. Composition of vitamin mixtures and amounts of vitamin supplements/100 g feed.

Vitamin or micro-nutrient	mg/100 ml water	mg/100 g feed
Vitamin mixture I:		
Thiamine hydrochloride . . .	40	1
Riboflavin	40	1
Pyridoxine hydrochloride . . .	40	1
Nicotinic acid amide	200	5
Folic acid	50	1.25
Menadione	100	2.5
Inositol	400	10
Calcium pantothenate	160	4
Vitamin mixture II:		
Choline chloride	4000	100
P-aminobenzoic acid	200	5

These vitamin mixtures have been in use for several years in nutritional studies on the rat at the Vitamin Department of the National Institute for Public Health, Stockholm. Their composition has been made known to me by Professor E. Brunius.

of vitamin B_{12} and 2.5 ml of each of two vitamin mixtures containing water-soluble vitamins and micronutrients (see Table 6). The fat-soluble vitamins were given in the poppy-seed oil (1 g of DL-α-tocopherol, 1,600,000 I.U. vitamin A, and 160,000 I.U. vitamin D_2 per kg oil). The diet thus contained 5 mg DL-α-tocopherol, 8,000 I.U. of vitamin A, and 800 I.U. of vitamin D_2 per 100 g. These vitamin supplements are several times greater than the requirements of the growing rat, as established by perusal of the literature (Brown and Sturtevant, 1949); the requirement of vitamin B_{12} under normal conditions is not known. If weanling rats are placed on a diet low in vitamin B_{12}, and containing iodized casein in order to stimulate metabolism, the growth-rate diminishes; on addition of B_{12} within the range 0.05–0.2 μg per rat per day the growth-rate increases in proportion to the amount administered (Register, Lewis, Thompson, and Elvehjem, 1949). Other vitamins were without effect (Lewis, Register, Thompson, and Elvehjem, 1949).

The synthetic, low-iron diet used in this investigation gave about 409 Calories per 100 g, and contained 250 μg of iron per 100 g. Cow's milk is stated to contain 200 μg of iron per 100 g, thus the iron per calorie in the synthetic diet amounted only to about 1/5 of that in cow's milk.

Iron added to the diet was given in the form of ferrous sulphate

which was diluted with lactose so that 100 mg of the mixture contained the desired quantity of iron. 100 mg of the different mixtures was weighed into gelatin capsules, and the contents of one of these capsules were mixed thoroughly with the daily ration.

Notes on the low-iron diet used in this work

Young rats weighing about 50 g reared on my low-iron diet developed within 2 months an iron-deficiency anaemia with a haemoglobin concentration of about 6 g per 100 ml. Animals reared on the same diet supplemented by adequate amounts of iron salts showed a haemoglobin concentration of about 16 g per 100 ml. This method of producing nutritional iron-deficiency anaemia has several advantages over the milk diet: rats grow much better than when on cow's milk alone; the diet is actually so complete that animals reared on it exhibit a high fertility; a pure iron deficiency is produced; it is possible to vary other ingredients in the diet, and to study the effect of this on the metabolism of iron.

No synthetic diet so low in iron that it produces a severe iron-deficiency anaemia in growing rats has previously been described in the literature.

Determination of the Haemoglobin Concentration

The haemoglobin was determined spectrophotometrically as oxyhaemoglobin, a method which has been recommended by many authors (Bell *et al.*, 1943, 1945; Szigeti, 1940; Lemberg and Legge 1949; and others). Lemberg and Legge obtained results of the same degree of accuracy as given by the manometric method. They carried out measurements on 1:100 dilutions of blood in 0.01 % sodium carbonate, and the extinction was read at 576 mμ. Szigeti warns against the use of alkaline solutions as strong as 0.4 % ammonia, since a certain amount of the oxyhaemoglobin is then converted to methaemoglobin within 1 hour. He recommends 0.04 % ammonia, and states that the ammonia should not be more dilute lest the proteins be precipitated. I have, however, been able to obtain clear solutions by diluting the blood 1:250 with 0.01 % ammonia. Most investigators have read the extinctions at one of the two absorption maxima of oxyhaemoglobin, viz. 576 or 540 mμ. The details of the procedure are as follows.

Procedure. 0.02 ml of blood was drawn into a micropipette and transferred to a test tube containing 5 ml of approx. 0.01 % ammonia. The pipette was rinsed several times with the solution. After thorough mixing the extinctions were read off in a Beckman spectrophotometer, model B, in 1 cm cuvettes at 540 mμ. The specimen was read against a blank

of the ammonia solution, prepared by diluting 0.5 ml of concentrated ammonium hydroxide (containing approx. 25 % NH_3) to 1 litre with distilled water. The extinctions of the blood dilutions remain constant for several hours.

Three determinations were done on each blood. The standard error of a single determination was calculated on the basis of triple determinations on 90 rats with a haemoglobin concentration of 12.2–18.4 g per 100 ml, and was found to be 0.34; the standard error of the mean of a triple determination was thus $\frac{0.34}{\sqrt{3}} = 0.20$.

The standard used was blood, the total iron of which had been determined after wet combustion.

Red Cell Counts, Haematocrit Readings, and Corpuscular Constants

The red cell counts were done according to Ellerman's method, in a Bürker's counting chamber.

The blood was diluted to 1:200 with Hayem's solution in an Ellerman tube, and the specimen mixed gently in an apparatus of the Spectram type. 16 CD-squares (0.02 mm^3) were counted, two counts being done on each blood. The standard error of a single determination was calculated from 60 double determinations on rat blood with a red cell count of 5–8.5 million per mm^3, and was found to be 0.23 million.

The haematocrit readings were done in graduated capillary tubes, 50 mm long and with an internal diameter of 1 mm. To prevent clotting, the capillary tubes were rinsed with 2 % heparin solution and dried in a thermostat at 37° C. The blood-filled tubes were centrifuged for 30 minutes in a Wifug centrifuge ("T-original") at 4800 r./minute. Double determinations were performed throughout. The standard error of a single determination, calculated from 60 double determinations on rat blood with a haematocrit reading of 35–49 %, was 0.7 %.

Some corpuscular constants defined. (See, for example, Wintrobe, "Clinical Haematology".)

Mean corpuscular volume (M.C.V.) in cubic microns

$$\frac{\text{haematocrit (vol. per cent)} \times 10}{\text{red cell count (mill. per mm}^3\text{)}}$$

Mean corpuscular haemoglobin (M.C.H.) in micromicrograms

$$\frac{\text{haemoglobin (g per 1000 ml)}}{\text{red cell count (mill. per mm}^3\text{)}}$$

Mean corpuscular haemoglobin concentration (M.C.H.C.) in per cent

$$\frac{\text{haemoglobin (g per 100 ml)} \times 100}{\text{haematocrit (vol. per cent)}}$$

Determination of Plasma Iron

The plasma iron was determined according to the method of Heilmeyer and Plötner (1937) as modified by Vahlquist (1941). Plasma had to be used for the determinations, since it was necessary to include the red blood cells in the determinations of total haemoglobin.

To begin with it was difficult to find a suitable anticoagulant effective in preventing clotting both at the rat's throat during blood-letting from the jugular veins and in the collecting tube. The anticoagulant had, in addition, to be iron-free and not interfere with the determinations of plasma iron. Heparin contains iron, and could not be freed of it by electrodialysis or cation exchange on resins. "Liquoid Roche" was found to contain no iron, and, in Vahlquist's method, had no influence upon the plasma iron determinations in quantities up to 10 mg per ml plasma: on addition of 20 mg per ml plasma the solutions became opalescent. Liquoid Roche was not effective in preventing clotting at the animal's throat. Since Hamilton, Gubler, Cartwright, and Wintrobe (1950) used potassium oxalate as anticoagulant in determining plasma iron this was also tried, but it was found to interfere with the iron *o*-phenanthroline reaction at the pH (about 2) which is used in Vahlquist's method: as little as 3 mg potassium oxalate per ml plasma reduced the plasma iron value from 310 to 96 µg per 100 ml. Hamilton *et al.* employed a method in which the pH was higher (about 4.1) than in Vahlquist's; and I have also confirmed that 3–14 mg potassium oxalate does not affect the iron *o*-phenanthroline reaction in their method.

A synthetic xylan polysulphuric acid, "Thrombocid"[1], was then tried as anticoagulant. Thrombocid is in common use in Germany (see Halse and Schmitz, 1949). It is free from iron, and had no influence upon the plasma iron determinations—at least not when used in amounts of up to 0.1 ml per ml plasma. It also effectively prevented coagulation at the throat during blood-letting.

In the end it became clear that the following technique was the most suitable for collecting the blood for plasma iron determinations. The skin on the ventral aspect of the neck was reflected, and the jugular veins exposed. About 0.3 ml of Thrombocid was applied to the exposed tissues, and the surplus swabbed away with cotton wool after a few

[1] Manufacturer: Dr. Willi Benend KG., München–Solln, Diefenbachstrasse 15. Thrombocid was kindly put at my disposal by Professor E. Jorpes, The Caroline Institute, Stockholm.

seconds. The jugular veins were opened, and 5–8 ml of blood was collected in a centrifuge tube containing about 10 mg of Liquoid Roche.

One ml of plasma was used for each determination, double determinations being, as a rule, performed. After 2 hours the extinctions were read off in 50 mm cuvettes in a Pulfrich Stufen-photometer with filter S 50. Blank values of 16–20 µg per 100 ml were obtained. The standard error of a single determination was calculated from 100 double determinations on plasma with a plasma iron level of 30–300 µg per 100 ml, and was found to be 4.2 µg per 100 ml.

Determination of Total Haemoglobin by Means of Viviperfusion

Principle. The animals were viviperfused according to the method of Copp and Greenberg (1946), except that the fluid was administered through the femoral vein and not the inferior vena cava. The whole perfusate was collected, the volume measured, and the haemoglobin content determined as oxyhaemoglobin on an aliquot of the perfusate.

Perfusion fluid and apparatus. A modified Tyrode solution of the following composition was used: NaCl 0.80 %, KCl 0.02 %, $CaCl_2$ 0.02 %, $MgCl_2$ 0.01 %, NaH_2PO_4 0.005 %, $NaHCO_3$ 0.10 %, and glucose 0.10 %. The two last-named were added immediately before use (Copp and Greenberg).

The solution was administered from a Mariotte bottle equipped with a 1.5 m long rubber tube, inner diameter 3 mm, with a long, fine, glass cannula at its free end, and fitted with a screw clamp for regulating the flow.

Procedure. The rats were anaesthetized with Nembutal-Abbott (sodium ethyl-(1-methylbutyl)-barbiturate). A 1:100 aqueous solution of Nembutal (5 mg/100 g body-weight) was injected intraperitoneally, and when it had taken effect the rat was bound in the dorsal position to an operating board. The right femoral vein was exposed and dissected free from the artery. Two silk ligatures were passed under the vein, which was then clamped with artery forceps high in the thigh. The exposed section of the vein was filled with blood by "milking" the vessels from the periphery, and the distal ligature tied. The glass cannula could now be introduced with comparative ease into this isolated, turgid section of the femoral vein lying in the middle of the field of operation. The skin was now reflected from the ventral surface of the neck, the salivary glands held aside, and the two branches of the external jugular vein dissected free on both sides. A kidney dish containing a few ml of 3.8 % sodium citrate was placed under the animal's head under a hole in the board.

The Mariotte bottle was filled with the fluid (warmed to 50° C), and placed 70 cm above the board. The rubber tube and cannula were rinsed with the solution and, when all air-bubbles had been expelled, the clamp was screwed tight. To prevent clotting when the jugular veins were opened 8–10 drops of Thrombocid were applied to the neck and any surplus swabbed away. The two branches of the jugular vein were opened on one side, and samples of blood taken for determination of haemoglobin, haematocrit reading, and red cell count. (Approximately 5 ml of blood was collected in a centrifuge tube containing about 10 mg of Liquoid Roche. The plasma was separated by centrifuging, and used for determining the plasma iron. The red blood cells were added to the perfusate.) The jugular veins of the other side were now opened.

The glass cannula was dipped in heparin and the screw clamp opened slightly while the cannula was introduced into the femoral vein.[1] The artery forceps was removed, and the cannula tied in with the upper ligature. 0.1 ml of heparin ("Heparin", Vitrum, 5 % solution) was injected into the tube immediately above the cannula, and the clamp opened almost fully. Diluted blood now began to drop more rapidly from the neck, to be collected in the dish with the citrate. The rate of perfusion was regulated with the clamp so that breathing and the action of the heart continued unimpeded as long as possible. The rat thus continued to breathe until the fluid emerging from the jugular veins was coloured only faintly pink. Perfusion was continued until the pure salt solution dropped from the neck. In a successful viviperfusion the heart would then still be beating. Carried out in this way the process was complete in 30–45 minutes.

As a rule the volume of the perfusate amounted to 300–400 ml. For determination of the haemoglobin content 0.5 ml of the perfusate was diluted with 5 ml of 0.01 % ammonia, and the haemoglobin determined spectrophotometrically as oxyhaemoglobin. If the perfusate was not clear it was filtered before carrying out this determination.

After perfusion most of the organs appear totally exsanguinated to the naked eye. The heart and coronary vessels contain a colourless fluid. The liver has a pale yellow-brown colour, and the portal vein contains pure perfusion fluid, as do the renal veins. The kidneys are pale greenish-brown, and the testes white. The mesentery and intestines are blood-free, the brain is white, and no blood is present in the meningeal vessels. On the other hand it is impossible completely to exsanguinate the bone-marrow and spleen: the marrow is coloured pink, and contains blood even after perfusion; the spleen is paler than usual, but still contains blood. It is thus clearly of importance to determine quantitatively the haemoglobin remaining after viviperfusion.

[1] The temperature of the perfusion fluid was about 38° C on entering the vein.

Determination of the Haemoglobin Remaining in the Animal after Viviperfusion

Ten viviperfused male rats weighing *c.* 300 g were used. The skeletal musculature of each rat was collected and weighed. Part was ground to a fine, pink powder with carbon dioxide snow, and the myoglobin and haemoglobin extracted with acetate buffer of pH 4.5 according to the method described by de Duve (1948) and Björk (1949). The extract was saturated with carbon monoxide, and the proportion of myoglobin and haemoglobin determined spectrophotometrically (de Duve, 1948): it was found that the haemoglobin amounted only to about 1 % of the amount of myoglobin. The remaining skeletal muscle tissue and the heart of each rat were homogenized in a Turmix. Of the homogenate 50 g was mixed with 50 ml of distilled water, hydrochloric acid was added until the pH was lower than 1, and the mixture allowed to stand for at least 15 minutes. The acid haematin was then extracted with 5 volumes of ether by shaking in a separating funnel; this process was repeated twice. The three ether extracts were mixed together and evaporated to a convenient volume. The extinctions were measured in 1 cm cuvettes in a Beckman spectrophotometer, model B, at a wavelength of 430 mμ. Blood with a known haemoglobin content, treated with hydrochloric acid followed by ether extraction of the haematin in the same way as the tissues was used as standard. The total amount of acid haematin which could be extracted from the muscles was found to correspond to 0.075–0.090 g myoglobin/100 g body-weight. Ignoring the iron-containing enzymes, the amount of haemoglobin remaining in the muscles after viviperfusion thus amounts to about 0.001 g/100 g body-weight. It is obvious that this is of no significance in the determination of the total haemoglobin.

In an approximate estimate of the amount of myoglobin in muscle the iron-containing enzymes may be ignored. In viviperfused muscle the greater part of these is made up of cytochrome c. Crandall and Drabkin (1946) state the amount of cytochrome c in rats to be 0.0056 g/100 g body-weight, of which about 80 % is found in the skeletal muscle. Theorell and Åkesson (1941) have shown that cytochrome c is so stable that it is impossible with certainty to distinguish spectroscopically cytochrome c that has been exposed to a pH of 1 from cytochrome c which has not. Its optical properties remain constant for several hours even in 2 N HCl.

According to Crandall and Drabkin, the myoglobin in the rat amounts to 0.04 g/100 g body-weight: this value is considerably lower than that calculated by me from the total quantity of haematin extracted from viviperfused muscle. Crandall and Drabkin, however, extracted the myoglobin with distilled water, which probably does not result in complete extraction.

After the muscles and heart had been removed from the carcase the remainder was homogenized in a mortar with carbon dioxide snow, hydrochloric acid was added to the homogenate, and the acid haematin extracted with ether. The liver and spleen were also subjected to this treatment. The haematin which was extracted from the liver, spleen, and body-remnants after removal of the muscles corresponded to 0.05–0.06 g of haemoglobin per rat. The greater part of this haematin probably derived from haemoglobin, the iron-containing enzymes being of no significance in this connection. As might be expected, most of the remaining haemoglobin (about 80 %) was found to be in the skeleton with its contained bone-marrow. The spleen contained 12–15 %. Only insignificant amounts of acid haematin could be extracted from the liver.

The total haemoglobin of these rats varied between 2.42 and 2.65 g. About 2–3 % of the total haemoglobin thus remains in the carcase, liver, and spleen after a successful viviperfusion.

Some remarks on the viviperfusion technique used in this investigation

Viviperfusion of rats has previously been used to obtain blood-free tissues (Austoni, Rabinovitch, and Greenberg, 1940; Copp and Greenberg, 1946), or in order to determine the total iron content of the blood (Gubler, Cartwright, and Wintrobe, 1950). No experiments have been done, however, to determine the amount of blood remaining in the animal after viviperfusion. My investigations have demonstrated that, with a careful technique, it is possible to eliminate practically all haemoglobin from the body. Direct determination of the total haemoglobin has the advantage over estimation from the blood volume and haemoglobin concentration that the source of error due to the fact that the haematocrit reading is considerably lower in the peripheral vessels than in the great veins is avoided. It has been shown that the total red cell volume is only about 75 % of the value obtained indirectly from the blood volume as determined by colour methods (Hahn, Ross, Bale, Balfour, and Whipple, 1942). Estimation of the blood volume by a colour method would in any case be inconvenient in this investigation, because the plasma is used for colorimetric iron determination.

In 20 normal male rats weighing 173–426 g I found the total haemoglobin determined by means of viviperfusion to be 0.74 ± 0.06 g Hb./100 g body-weight. Garcia, Van Dyke, and Berlin (1952) report values between 0.58 and 0.83 g Hb./100 g body-weight for 4 normal female rats in which the red cell volume was estimated with the aid of red cells labelled with Fe^{59}. Crandall and Drabkin (1946) obtained a considerably higher value for the total haemoglobin in rats, viz. 1.27 g Hb./100 g body-weight: they calculated this from the blood volume determined with T-1824 and the haemoglobin concentration, with the reservation that their figure for the blood volume is only approximate.

I also calculated the total red cell volume of the above rats from the total haemoglobin and the figures for haemoglobin concentration and haematocrit (jugular vein): the total red cell volume was found to be 2.14 ± 0.17 ml/100 g body-weight. Berlin, Huff, Van Dyke, and Hennessy (1949) used erythrocytes labelled with P^{32} to estimate the total red cell volume in the normal rat, and found it to be 2.16 ± 0.20 ml/100 g body-weight. My figures for the total red cell volume thus correspond closely with those obtained with the aid of radio-active isotopes.

Determination of Dry Weight, Protein, and Total Fat of the Carcase

By carcase is meant the body of the viviperfused animal after removal of the skin, liver, spleen, and the digestive tract.

The whole carcase was homogenized in a Turmix without the addition of water, and about 50 g of the resulting pulp weighed out and treated twice with 10 volumes of acetone and twice with 10 volumes of ether. In this way the carcase was reduced to a fine powder with a water content of about 5 %, most of the fat having been extracted. The water content of this powder remains constant for several weeks on storage in stoppered vessels. The powder was weighed, and used for quantitative determination of the non-haemin iron and nitrogen. The nitrogen was determined according to Kjeldahl's macro-method, the protein content then being calculated by multiplying the nitrogen value by 6.25. In the modification of Kjeldahl's macro-method used the sample is digested in boiling, concentrated sulphuric acid, small pieces of copper wire being added as catalyst. Digestion is continued until the solution has a clear blue or green colour, when boiling is interrupted and the solution cooled to room-temperature. $KMnO_4$ is then added, and boiling resumed for a further hour. The standard error of a single determination, calculated from double determinations, was found to be 0.15 % for a nitrogen content of about 12 % in the carcase-powder.

When the total fat in the carcase was to be determined the powder was extracted with a 3:1 mixture of alcohol and ether as described by Bloor (1915). The acetone, ether, and alcohol-ether extracts were evaporated to dryness, and the fat dissolved in chloroform and weighed after evaporation of the chloroform.

The dry weight of the defatted powder was estimated from an aliquot dried to constant weight *in vacuo* over phosphorus pentoxide at 100° C. Since the carbohydrate level in the tissues is low, an approximate value for the salts can be obtained by subtracting the quantity of protein from this dry weight.

The dry weight of the liver, and its protein and fat contents, were determined by the method described for the carcase. The liver, however, was homogenized in a mortar with the aid of glass knives.

Statistical Methods

Owing to the small number of animals in each group the *t*-test worked out by Gosset and Fisher (see Fisher, 1946) has been used in comparing the means. The significance is denoted by P, which signifies the probability of a difference at least as large as that observed arising by chance. The following significance levels have been used. The difference is termed highly significant where $P < 0.001$, significant where $0.001 < P < 0.01$, and probably significant where $0.01 < P < 0.05$ (see Cramér, 1951; and others).

The standard error of a single determination (s) was calculated from n double determinations, according to the formula $s = \sqrt{\frac{d^2}{2n}}$ (see Dahlberg, 1940), where d^2 is the sum of the squares of the differences between the two determinations in each pair. In calculating the standard error of a single determination from n triple determinations with the standard deviations s_1, s_2, $\cdots$ s_n the more general formula

$$s = \sqrt{\frac{s_1^2 + s_2^2 + \cdots s_n^2}{n}}$$

was used (see Arley and Buch, 1943).

The coefficient of partial correlation between the variates x_1 and x_2 after elimination of a third variate x_3 ($= r_{12.3}$) was obtained from the formula

$$r_{12.3} = \frac{r_{12} - r_{13} \times r_{23}}{\sqrt{(1 - r_{13}^2)(1 - r_{23}^2)}}$$

CHAPTER II

Haemoglobin Formation in Protein Deficiency

Review of the Literature

Before describing my own experiments it will be appropriate here to give a critical review of the earlier work concerning the influence of protein deficiency on haemoglobin formation.

Animal experiments on haemoglobin formation in protein deficiency include those where the animals were kept on a low-protein diet and those in which an essential amino-acid was excluded.

Whipple *et al.* studied the formation of haemoglobin and plasma proteins in dogs with experimental anaemia and hypoproteinaemia produced by bleeding the animals and feeding them on a low-protein diet. It was found that, in the presence of this double depletion (anaemia + hypoproteinaemia), the organism utilizes protein from tissues and organs in the formation of haemoglobin and plasma proteins, the circulating proteins thus having priority over the proteins of the tissues and organs. The animals lose weight, and as a rule the larger the amount of blood let the greater the production of blood proteins and the greater the weight loss. When Whipple *et al.* attempted to produce anaemia without hypoproteinaemia by bleeding dogs kept on a low-protein diet, the animals suffered at the same time a rapid loss of plasma owing to the blood-lettings, and this soon led to the double depletion mentioned above. If the plasma was retransfused it soon became obvious that the plasma proteins were used in the formation of new haemoglobin, and the anaemia diminished. When more protein or a mixture of essential amino-acids was administered to the dogs, still keeping them in a state of anaemia and hypoproteinaemia, the production of haemoglobin was always greater than the production of plasma proteins: usually 2–4 g of haemoglobin was formed for every gram of plasma protein. It is obvious that, in these experiments, haemoglobin formation took high priority in the competition for material for the building up of protein.

Weech, Wollstein, and Goettsch (1937) studied the changes in the red cell mass in dogs on a low-protein diet. The plasma volume was determined with vital red or T-1824, and the red cell mass calculated from the plasma volume and the haematocrit reading for the great veins. By 80 days the red cell mass had fallen to 53 % of the initial value,

and the body-weight to about 80 % (*N.B.* the dogs had oedema). Weech *et al.* calculated that an average of 18.7 % of the protein lost during the experiment was haemoglobin. The haemoglobin concentration fell during the period of the experiment to 80 % of the initial value. In this work, however, there is no means of calculating the percentage decrease in body protein.

Orten and Orten (1943) produced a slight anaemia in rats by placing them, after weaning, on a diet containing 3.5 % lactalbumin: the controls received 18 % lactalbumin ("adequate-protein" group). After a period of 76 days the haemoglobin concentration in the low-protein group was 11.5 g per 100 ml, and in the adequate-protein group 15.8 g per 100 ml: the red cell count was the same in both groups, the anaemia thus being of "hypochromic" type. The animals in the low-protein group consumed slightly more than half as much food as the controls. One group of rats on the same calorie intake as the low-protein animals, but receiving the same amount of protein as the adequate-protein animals, had a normal haemoglobin level; and the animals receiving a diet containing 18 % lactalbumin in amounts corresponding to the food consumption in the low-protein group had an almost normal blood picture. The lowest haemoglobin levels (9.9 g per 100 ml) were exhibited by four animals that were given protein as in the low-protein group, but which were force-fed with sugar and fat until the calorie intake was equal to that in the adequate-protein group. Orten and Orten were able to cure low-protein anaemia in four weeks by administering extra protein without changing the intake of calories, iron, or other ingredients in the feed. An increase in iron intake by 1 mg of iron per day had no permanent beneficial effect on the low-protein animals: a slight transient increase in the haemoglobin concentration was obtained, but at the end of the experiment the haemoglobin level was the same as in the animals without extra iron. The work of Orten and Orten proves without doubt that the anaemia in the low-protein animals was due to inadequate comsumption of protein rather than to too low calorie intake.

Metcoff, Favour, and Stare (1945) found that the haemoglobin concentration in growing rats reared on a diet containing 8 % casein was not diminished after a period of 10–14 weeks. The controls received a diet containing 18 % casein. Metcoff *et al.* estimated the plasma volume by means of T-1824, and calculated the blood volume and red cell mass from the plasma volume and haematocrit reading. The total circulating haemoglobin was calculated and converted to unit value (g haemoglobin per unit blood volume). The unit value for the blood volume was obtained by placing the total volume in relation to a unit of body-surface (ml blood per 100 cm^2). The unit circulating haemoglobin and the unit blood and cell volumes were significantly diminished after 10–14 weeks on an 8 % casein diet.

Buechler and Guggenheim (1949) fed male rats weighing 150–250 g on a protein-free diet for 8–10 weeks. The rats diminished in weight 28–35 %, and developed moderate anaemia. The haemoglobin concentration fell to 8.5 g per 100 ml compared with 14.4 g per 100 ml in the controls. If the protein-deficient animals were given a diet with 3, 9, or 18 % of casein *ad lib.* they increased in weight, and the haemoglobin concentration rose, more or less in proportion to the casein content of the diet. Two groups of protein-depleted animals were fed on a diet containing 9 and 18 % casein respectively, but they were given only 60 % of the quantity they consumed when they were allowed to feed freely: the haemoglobin concentration rose as it did when the same diet was given *ad lib.*, but the increase in weight was considerably less. The authors draw the conclusion that a calorie intake low enough to prevent normal increase in weight results in no reduction in haemoglobin formation. They hold that these findings corroborate the results of Whipple *et al.* concerning the priority of haemoglobin formation over the proteins of the tissues and organs. In this connection it must be objected that one has to take account of changes in the total haemoglobin before drawing conclusions on order of priority: it is not certain that the increase in total haemoglobin is greater than the general increase in body proteins in these experiments with restricted calorie intake.

Shemin and Rittenberg (1946) have demonstrated that glycine is a precursor in the protoporphyrin synthesis. When the dietary protein is low it is possible that haemoglobin formation might be limited owing to lack of material for the building up of protoporphyrin. Orten and Keller (1946) determined the faecal excretion of protoporphyrin in growing rats on a low-protein diet (3.5 % casein) and on a diet containing 22.5 % casein. It was consistently much lower in the low-protein animals than in the controls, whether calculated as micrograms per 0.5 g of dried faeces, as micrograms per rat per day, or as micrograms per 100 g body-weight per day; but it was significantly greater in the low-protein animals when expressed as micrograms of protoporphyrin per gram of ingested protein. Orten and Keller believe that the faecal excretion of protoporphyrin can be taken as a measure of porphyrin synthesis in the body . They consider that, if this is true, their findings prove that the dietary protein is a precursor of protoporphyrin in the rat, and that porphyrin formation, like haemoglobin formation, takes a high priority in the competition for protein in the organism. It is clear that the quantity of synthesized protoporphyrin per gram of ingested protein will be relatively low on high protein intake, since a large proportion of any protein surplus is used for combustion and not for the synthesis of protein and other substances.

Ten amino-acids have been shown to be indispensable for optimum growth in the young rat (Rose, 1938). These are methionine, threonine, phenylalanine, tryptophan, histidine, leucine, isoleucine, valine, lysine,

and arginine, all of which, with the exception of arginine, have been found to be indispensable in the maintenance of appetite, weight, and nitrogen balance in the adult rat (Wissler *et al.*, 1948). The effect of the absence from the diet of rats of one of these essential amino-acids upon the haemoglobin concentration has been studied for each of them, and the effect upon the red blood cells has been examined for some of them.

Albanase *et al.* (1943) examined the effect of tryptophan deficiency upon the haemoglobin concentration and plasma proteins in rats. Seven adult male rats were kept on a diet in which the protein consisted of acid-hydrolyzed casein supplemented with cystine. Within a period of 86–134 days five of the animals had developed a slight anaemia with haemoglobin levels of 11.4–12.6 g per 100 ml; the haemoglobin concentration in the controls was 13.3 ± 0.34 g per 100 ml. The fall in the haemoglobin level was not followed by a reduction in the number of red blood cells. Two of the tryptophan-deficient animals which had been on the deficiency diet for 64 and 86 days respectively developed no anaemia, probably because of the relatively short experimental period. Trials with younger rats gave similar results. A group of rats on tryptophan-supplemented diet limited to the amount consumed by the tryptophan-deficient animals had normal haemoglobin values.

Albanese *et al.* (1946) have since investigated the effect upon the haemoglobin concentration of a cystine-deficient diet and of a diet poor in both cystine and methionine. The former had no ill effect upon the haemoglobin concentration; but rats kept on the latter diet developed hypoproteinaemia and anaemia with a haemoglobin concentration, after a period of 90–150 days, of 11.5 g per 100 ml (controls 15.8 g per 100 ml). The protein fraction of the diet deficient in methionine and cystine was prepared by tryptophan reinforcement of a hydrogen peroxide-oxidized acid hydrolysate of casein. The marked drop in haemoglobin caused by the deficient diet was not accompanied by a fall in the red cell count. Dent and Rimington (1947) have, however, shown that oxidized casein is toxic and causes porphyrinuria in rats, probably owing to the presence of methionine sulphone.

Glynn, Himsworth, and Neuberger (1945) reared growing rats on a synthetic diet containing an amino-acid mixture instead of protein. When both cystine and methionine were excluded from the mixture the animals developed severe anaemia which was often hyperchromic and macrocytic. The animals, however, also developed massive liver necrosis, probably because of the deficiency of vitamin E in the diet.

The two last-named investigations into the effect of methionine deficiency upon haemoglobin formation are thus not without cause for objection.

Gillespie, Neuberger, and Webster (1945) found that lysine deficiency in rats resulted in slight anaemia with a somewhat greater diminution

in the haemoglobin concentration than in the number of red cells. The dietary protein was administered in the form of zein supplemented by tryptophan.

Maun *et al.* (1945–46) carried out comprehensive morphological studies on growing rats reared on a synthetic diet containing a mixture of essential amino-acids from which one of the amino-acids phenylalanine, leucine, or histidine had been excluded. The haemoglobin concentration and plasma proteins were also determined. When phenylalanine was lacking the animals developed moderate anaemia with haemoglobin levels of between 7.4 and 14.0 g per 100 ml (mean 9.9 g per 100 ml); controls on the same diet supplemented by phenylalanine had haemoglobin values of 13.7–15.8 g per 100 ml (mean 14.7 g per 100 ml). Leucine deficiency was not followed by any fall in the haemoglobin concentration. In the histidine-deficient animals the haemoglobin concentration was consistently lower than in the controls (means 10.4 and 14.1 g per 100 ml respectively). The experimental period in these experiments was 28 days. Fuller, Neuberger, and Webster (1947) also report the finding of slight anaemia in rats kept on a diet containing a histidine-free mixture of amino-acids. In none of these investigations concerned with deficiency of phenylalanine, leucine, and histidine was the number of red cells determined.

In none of the investigations on amino-acid deficiency mentioned above was the state of the total haemoglobin a subject for scrutiny.

Benditt *et al.* (1947) studied the formation of plasma proteins and erythrocytes on administration of amino-acid mixtures to protein-depleted rats. The plasma volume was estimated with the aid of T-1824. A mixture of the nine amino-acids which are indispensable to the adult rat produces equally great regeneration of red blood cells as a diet containing 9 % of casein. If one of these amino-acids is missing no significant increase in the red cell mass takes place. On the other hand, a rat on a synthetic diet containing an amino-acid mixture completely lacking in arginine is able to form new erythrocytes to the same extent as one on a diet containing casein or an amino-acid mixture in which all the essential amino-acids, including arginine, are present.

Summing up the results of the investigations into the effect upon haemoglobin formation of the absence of an essential amino-acid from the diet it would appear that lack of one of these is usually followed by moderate anaemia with a fall in the haemoglobin concentration amounting to 20–30 %. In those cases where both the red cells and the haemoglobin were estimated the anaemia has most commonly been "hypochromic".

No investigations into the effect of pure protein deficiency on haemoglobin formation have been done in man, but the effects of starvation and semi-starvation have often been examined. In most of these latter investigations only the haemoglobin concentration has been determined,

the red cell count occasionally having been done. A comprehensive work on the effect of semi-starvation upon the human organism, including haemoglobin balance, has been published by Keys *et al.* (1950, "The Minnesota Experiment"). Thirty-two healthy young men were placed for 24 weeks on a diet resembling as closely as possible that of the starvation areas of Europe during the Second World War. The average daily ration gave 1570 Calories, and contained *c.* 50 g of protein (chiefly vegetable) and 30 g of fat: the daily intake of animal protein varied between 5.3 and 8.4 g. The subjects were examined before the experiment, during the period of semi-starvation, and during the recovery period: among the many investigations done were determinations of haemoglobin, red cell count, haematocrit reading, and the blood volume. Initially the haemoglobin concentration was 15.1 ± 0.88 g per 100 ml, the red cell count 5.22 million per mm^3 blood, and the colour index 0.98. During the 24 weeks of semi-starvation the haemoglobin concentration fell: at 12 weeks it was 12.6 ± 0.8 g per 100 ml, and at 24 weeks 11.7 ± 0.8 g per 100 ml (total fall 22.6 %). After 24 weeks the red cell count had fallen to 3.78 million, a fall of 27.6 %, and during the same period the colour index rose by 14.3 % from 0.98 to 1.11. The haematocrit reading fell to the same extent as the haemoglobin. That the fall in the red cell count was greater than the fall in haemoglobin and haematocrit values proves that the anaemia was of macrocytic type.

The total haemoglobin, calculated from the blood volume (estimated with T-1824) and the haemoglobin concentration, diminished during the 24 weeks from 890 g to 631 g, a fall of 29.0 %. The body-weight diminished by 23.8 %. The "active tissue" was calculated as the difference between the body-weight and the sum of fat, extracellular fluid, and bone minerals, and was found to have diminished by 27.3 % during the period of semi-starvation. The percentage loss of total haemoglobin was thus greater than the percentage fall in body-weight, and was similar to the percentage loss of active tissue.

The anaemia produced in the Minnesota experiment was plainly nutritional in character. It was not of iron deficiency type. The dietary iron (12.2 mg per day) appears to have been adequate, and the subjects may be assumed to have had normal iron stores at the beginning of the experiment. The diet contained extremely little animal protein, but it must not be concluded that the anaemia was due purely to protein deficiency. The diet was also low in vitamins, among them the important blood factors vitamin B_{12} and nicotinic acid; and a latent nicotinic acid deficiency can become manifest in the presence of tryptophan deficiency, a lesson learnt in the pellagra districts. It is thus impossible to say to what extent individual factors in the diet have contributed to the development of the nutritional anaemia of the Minnesota experiment.

Summarizing the results of earlier investigations into the effect of protein deficiency upon haemoglobin synthesis the following may be said. Haemoglobin balance during protein deficiency has not been the object of systematic study under various experimental conditions in animals not subjected to blood-letting. There are several investigations which show that the *haemoglobin concentration* is slightly reduced in severe protein deficiency, but I have found only two investigations in which the *total haemoglobin* was examined in animals not bled. In one of these it is shown that, as might be expected, the total haemoglobin falls in connection with the great weight loss resulting from severe protein deficiency; and in the other it is shown that in growing animals the quantity of haemoglobin per unit body surface is reduced. It is plain that, in studying the position of haemoglobin in the competition for material for protein synthesis when this is in short supply, the chief point of interest lies in the changes in total haemoglobin in relation to the other body proteins. No such investigations have previously been described.

In all previous experiments on haemoglobin formation during protein deficiency the animals have been given a surplus of iron: in none has the effect upon haemoglobin formation of simultaneous deficiency of both protein and iron been studied.

CHAPTER III

Haemoglobin Formation and Storage Iron in Adult Male Rats on Varying Protein Intake

This first experiment was carried out to examine quantitatively the state of the total haemoglobin and the storage iron in adult rats during protein deficiency. No iron was given over and above the extremely small quantities present in the synthetic diet, in order to simplify the study of the quantitative changes in the storage iron accompanying changes in the total haemoglobin.

Experimental

Twenty-four 4-month-old male rats were arranged in four equal groups. The corresponding animals in the different groups belonged to the same litter. The body-weight at the start was 294–373 g, and the mean weights of the various groups were almost identical (336, 339, 333, 333). The animals were placed in individual cages of chromium-plated brass netting, this material being selected to prevent the animals from getting any iron by gnawing the cages. Group I was fed on a diet containing 18 % of spray-dried hen's egg-white, Group II receiving 9 %, Group III 4.5 %, and Group IV 2.25 % of the same protein. Each animal was offered a daily ration of 18 g of feed, weighed into a glass vessel. The ration was consumed in its entirety by the animals of Groups I–III, whereas those in Group IV consumed less. Groups I–III were thus reared isocalorically, the only difference between them lying in the intake of protein and cane sugar. The calorie content of this diet is 409 Calories per 100 g. The animals in Groups I–III thus received 22 Calories per 100 g body-weight at the beginning of the experiment. The consumption of protein calculated as mg of nitrogen per 100 g body-weight per day was, at the beginning of the experiment, 154 mg in Group I, 77 mg in Group II, and 38.5 mg in Group III. The iron content of the diet is 2.5 μg per gram, the daily iron intake of each rat in Groups I–III thus being 45 μg. The drinking water was ordinary tap water, which was found to be practically iron-free; it was supplied in glass drinking flasks. The experimental period varied in duration be-

tween 47 and 134 days, and in Groups I–III was adjusted so that the means for each group were approximately the same (96, 99, and 93 days). In Group IV the experimental period was shorter (mean 63 days), for fear the animals should die of protein deficiency; the animals in this group grew weak and tired, and the fur rough.

At the end of the experiment the following determinations were carried out on each of the animals: haemoglobin concentration; total haemoglobin; plasma iron; non-haemin iron in the liver, spleen, and carcase; protein and fat in the carcase; and protein and fat in the liver. In addition certain organs were weighed, viz. liver, spleen, kidneys, testes, and adrenals. The carcase consisted of the exsanguinated animal after removal of skin, alimentary tract, liver, and spleen. The haemoglobin concentration, total haemoglobin, plasma iron, non-haemin iron, and protein in the carcase are given in Table 7 a; and the body-weights, organ-weights, and the percentage composition of carcase and liver are collected in Table 7 b.

Results

During the first part of the experiment the animals in all groups diminished in weight: at 6 weeks the loss in weight expressed in per cent of the initial weight was 4.8 % in Group I, 5.9 % in Group II, 16.2 % in Group III, and 24.9 % in Group IV. The difference in weight at 6 weeks between Group III and the combined Groups I and II was highly significant. At the end of the experiment the Group III animals had approximately regained their initial weight, whereas the body-weight in Group I exceeded the initial weight by 12.2 % and in Group II by 7.4 %. The weight in Group III at the end of the experiment was 10.6 % lower than that in Group I, the difference being probably significant. In Group IV there was hardly any change in weight after 6 weeks: the mean body-weight at the end of the experiment (47–91 days) was 254 g, compared with 250 g at 6 weeks.

The *haemoglobin concentrations* in Groups I and II were approximately the same (means 15.2 and 15.7 g per 100 ml respectively). The somewhat lower mean for Group I was due to one of the animals (rat no. 1) having a lower haemoglobin concentration than the rest, and lower than that usually seen in healthy adult rats. The haemoglobin concentration in Group III was 14.3 g per 100 ml, somewhat lower than in Groups I and II: the difference between Group III and the combined Groups I and II, however, is not significant. The series is unfortunately too small to conclude whether 4.5 % of spray-dried hen's egg-white in the diet is inadequate for maintaining an entirely normal haemoglobin concentration. The average haemoglobin concentration in Group IV was 12.9 g per 100 ml (here, too, the first rat in the group

Table 7 a. Haemoglobin, non-haemin iron, and protein in the carcase of adult male rats on different protein intakes.

Rat No.	Days on experiment	Hb. concentration, g per 100 ml	Total haemoglobin, g	Plasma iron, µg per 100 ml	Non-haemin iron in liver, mg	Non-haemin iron in spleen, mg	Non-haemin iron in carcase, mg	Total non-haemin iron, mg	Protein in the carcase, g	g Hb. per g protein in the carcase
					Figures in brackets indicate mg non-haemin iron per 100 g fresh tissue					
	Group I. 18% protein in the diet.									
1	134	13.5	2.02	129	0.150 (0.89)	0.030 (4.3)	4.04 (1.33)	4.22	42.0	0.048
2	55	15.0	2.38	163	1.209 (7.14)	0.227 (22.5)	3.16 (1.26)	4.60	41.0	0.058
3	76	15.2	2.59	294	0.573 (3.70)	0.705 (69.0)	3.48 (1.22)	4.76	44.2	0.059
4	104	14.8	2.43	156	0.195 (1.11)	0.104 (10.1)	2.51 (0.87)	2.81	43.0	0.057
5	125	16.0	2.97	258	0.158 (0.84)	0.073 (7.0)	3.40 (1.10)	3.63	44.3	0.067
6	84	16.8	2.33		0.787 (5.50)	0.577 (82.2)	3.19 (1.27)	4.55	43.7	0.053
Mean	96	15.2	2.45	200	0.512 (3.20)	0.286 (32.5)	3.30 (1.18)	4.10	43.0	0.057
	Group II. 9% protein in the diet.									
7	123	14.8	2.18	192	0.163 (1.05)	0.049 (7.2)	3.78 (1.24)	3.99	42.3	0.052
8	74	15.8	2.25	178	0.456 (2.96)	0.117 (12.9)	3.38 (1.34)	3.95	39.3	0.057
9	79	16.6	2.31	259	0.288 (2.28)	0.087 (10.2)	2.71 (0.99)	3.09	47.4	0.049
10	109	15.8	2.57	288	0.176 (1.15)	0.054 (5.6)	2.46 (0.80)	2.69	45.4	0.057
11	119	15.3	2.37	176	0.172 (1.00)	0.068 (6.4)	2.83 (1.03)	3.07	38.1	0.062
12	91		2.22		0.296 (2.37)	0.256 (25.6)	3.00 (1.30)	3.55	42.8	0.052
Mean	99	15.7	2.32	219	0.259 (1.80)	0.105 (11.3)	3.03 (1.12)	3.39	42.6	0.055
	Group III. 4.5% protein in the diet.									
13	117	13.8	2.01	133	0.498 (2.82)	0.055 (7.6)	4.37 (1.62)	4.92	37.2	0.054
14	61	14.3	1.86	105	1.590 (9.28)	0.806 (111.1)	2.94 (1.26)	5.34	34.3	0.054
15	111	13.9	2.14	195	1.437 (7.68)	0.381 (55.9)	2.78 (0.87)	4.60	46.9	0.046
16	113	16.0	2.36	293	0.550 (3.82)	0.241 (28.3)	3.46 (1.17)	4.25	39.8	0.059
17	71	12.1	1.67	140	1.547 (8.13)	0.214 (30.1)	3.07 (1.33)	4.83	36.1	0.046
18	82	15.7	2.26	304	1.282 (7.73)	0.600 (70.6)	3.61 (1.42)	5.49	37.1	0.061
Mean	93	14.3	2.05	195	1.151 (6.58)	0.383 (50.6)	3.37 (1.28)	4.91	38.6	0.053
	Group IV. 2.25% protein in the diet.									
19	47	8.7	1.24		0.586 (5.03)	0.640 (82.7)	3.48 (1.76)	4.71	23.5	0.053
20	50	13.6	1.63	184	1.252 (7.15)	0.463 (91.9)	4.28 (2.34)	6.00	29.5	0.055
21	58	12.9	1.40	165	2.368 (12.16)	0.839 (117.9)	4.85 (2.39)	8.06	33.4	0.042
22	68	13.5	1.54	216	1.842 (9.38)	1.181 (167.9)	3.61 (1.67)	6.63	37.1	0.041
23	91	14.2	1.56		0.828 (7.05)	0.828 (65.9)	3.65 (1.87)	5.31	31.5	0.050
24	63	14.2	1.60	141	2.384 (13.30)	1.448 (215.6)	3.68 (1.86)	7.51	32.0	0.050
Mean	63	12.9	1.50	177	1.543 (9.01)	0.900 (123.7)	3.93 (1.98)	6.37	31.2	0.049

Table 7 b. Body-weight, weight of organs, and composition of carcase and liver of adult male rats on different protein intakes.

Rat No.	Body-weight at beginning of experiment, g	Body-weight at end of experiment, g	Weight of carcase, g	Protein in carcase, %	Fat in carcase, %	Salts and carbohydrates in carcase, %	Weight of liver, g	Fat in liver, %	Protein in liver, %	Salts and carbohydrates in liver, %	Weight of spleen, g	Weight of kidneys, g	Weight of testes, g	Weight of adrenals, mg
Group I. 18% protein in the diet.														
1	299	392	304	13.9	21.0	4.9	16.81	4.7	10.4	1.8	0.71	3.88	1.79	38.8
2	340	360	253	16.2	8.0	6.4	16.93	3.5	11.2	1.8	1.01	—	2.15	—
3	373	387	285	15.5	7.3	6.6	15.49	4.4	8.8	1.3	1.02	3.93	2.40	41.6
4	339	365	288	15.0	13.6	4.8	17.60	0.9	10.6	1.1	1.03	3.63	2.52	39.9
5	331	419	309	14.3	13.1	6.0	18.85	4.8	12.7	2.1	1.05	4.20	2.63	44.6
6	332	338	251	17.4	8.7	6.4	14.30	4.1	12.2	1.3	0.70	3.92	2.75	42.0
Mean	336	377	282	15.4	12.0	5.9	16.66	3.7	11.0	1.6	0.92	3.91	2.37	41.4
Group II. 9% protein in the diet.														
7	294	384	305	13.9	16.3	5.5	15.54	5.0	11.9	1.4	0.69	3.64	2.53	35.0
8	310	341	252	15.6	8.4	5.6	15.39	3.6	8.8	1.7	0.91	3.77	2.57	37.7
9	369	366	274	17.3	7.6	6.4	12.62	4.8	15.0	1.1	0.85	4.26	2.23	40.2
10	358	370	308	14.7	6.3	5.6	15.29	3.7	9.3	0.8	0.97	3.61	2.53	49.2
11	347	387	275	13.9	11.4	5.8	17.22	4.6	10.9	2.3	1.05	3.57	2.63	34.6
12	357	338	231	18.5	6.6	8.3	12.48	4.4	9.1	1.0	1.00	4.14	2.70	41.4
Mean	339	364	274	15.7	9.4	6.2	14.76	4.4	10.8	1.4	0.91	3.83	2.53	39.7
Group III. 4.5% protein in the diet.														
13	330	331	270	13.8	12.4	5.1	17.64	5.8	9.3	1.2	0.72	2.94	2.73	32.6
14	310	306	233	14.7	13.4	6.1	17.13	10.0	8.3	1.5	0.73	2.54	1.44	23.7
15	373	386	320	14.6	16.2	4.2	18.70	8.3	9.2	3.5	0.68	3.53	2.15	33.2
16	337	350	296	13.5	13.3	6.1	14.40	6.0	9.7	1.9	0.85	3.34	2.27	34.1
17	301	304	231	15.6	10.8	6.4	19.02	7.4	8.0	0.9	0.71	2.40	2.26	21.5
18	344	343	254	14.6	12.1	5.2	16.58	4.5	9.3	1.2	0.85	3.52	2.36	36.5
Mean	333	337	267	14.5	13.0	5.5	17.25	7.0	9.0	1.7	0.76	3.05	2.20	30.3
Group IV. 2.25% protein in the diet.														
19	301	226	198	11.9	4.3	7.5	11.65	9.6	6.8	1.1	0.77	—	—	—
20	315	256	183	16.1	12.4	6.5	17.50	10.4	6.6	3.2	0.50	—	1.00	—
21	352	270	203	16.4	12.2	6.8	19.48	5.3	8.3	4.2	0.71	2.75	2.09	—
22	364	273	216	17.2	9.1	5.1	19.64	5.9	7.3	2.7	0.53	2.37	1.59	31.7
23	333	236	195	16.2	8.4	6.1	11.75	8.0	7.9	1.5	0.70	2.29	1.64	23.8
24	335	265	198	16.1	12.0	5.4	17.92	6.9	7.5	1.7	0.67	2.13	1.85	—
Mean	333	254	199	15.7	9.7	6.2	16.32	7.7	7.4	2.4	0.65	2.39	1.63	—

differed from the others in having a considerably lower haemoglobin concentration). The difference in haemoglobin concentration between the combined Groups I and II (mean 15.4 g per 100 ml) and Group IV (mean 12.9 g per 100 ml) is probably significant. If the deviating first rat is excluded, the mean 13.7 g per 100 ml is obtained for the other animals in Group IV: the fall in haemoglobin concentration in Group IV is now highly significant. Thus, in adult male rats, cutting down the protein content of the diet to 2.25 % of spray-dried hen's egg-white is followed, after an experimental time of 47–91 days, by a fall of 10–15 % in the haemoglobin concentration.[1]

The average *total haemoglobin* value was approximately the same for Groups I and II (2.45 and 2.32 g respectively), but for Group III it was lower (2.05 g): the decrease amounts to 16.3 % of the total haemoglobin value for Group I, and is probably significant. Finally, in Group IV the total haemoglobin was only 1.50 g, i.e. 38.8 % lower than in Group I, the difference being highly significant. By expressing the haemoglobin concentration and total haemoglobin for each of the animals in Groups III and IV in per cent of the corresponding means for Group I, it can be determined whether the percentage decrease in total haemoglobin is significantly greater than the percentage decrease in haemoglobin concentration: in Group III it is not significant, but in Group IV it is significant. Thus when the protein content of the diet is inadequate the total haemoglobin is affected more than the haemoglobin concentration. This is demonstrated in Fig. 4.

To examine the effect of protein deficiency on the total haemoglobin in relation to the other body proteins in general we make use of *the quotient g Hb. per g carcase protein.* As can be seen from Table 7 a, this quotient shows a tendency to fall with diminishing protein intake, but the differences between the groups are not great. In Group IV the quotient is *c.* 14 % lower than the value in Group I, the difference being probably significant. If the protein content of the diet is small enough the total haemoglobin thus diminishes more than the other body proteins in general.

To get a more complete picture of the position of haemoglobin formation in the competition for material for protein synthesis when this is in short supply, I have sought for other signs of protein deficiency present at the same time as a notable decrease in the total haemoglobin. In this connection the conditions in the third group, with 4.5 % of protein in the diet, are of particular interest: firstly the calorie consumption is the same in this group as in Groups I and II, and secondly it is not until this group that any decrease in total haemoglobin can be established. Since the animals in Group III increased in weight during the

[1] When an increase or decrease is expressed in per cent this always implies per cent of the corresponding mean for the animals which are taken as controls.

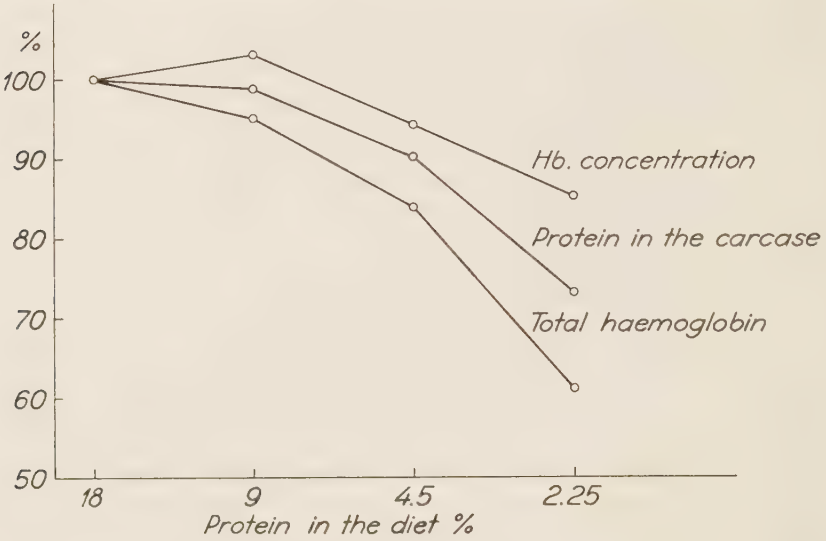

Fig. 4. Haemoglobin concentration, total haemoglobin, and total carcase protein of adult male rats on different protein intakes.

latter part of the experiment they were able, in spite of the low protein intake, to achieve positive nitrogen balance, at least after a certain period of acclimatization. In these animals it is evident that the shortage of protein limited haemoglobin formation to at least the same extent as the body-weight and the total amount of protein in the carcase.

It is known that animals kept on a low-protein diet develop fatty livers (Wang *et al.*, 1949; and many others). It is possible in this experiment to ascertain whether a decrease in protein intake just enough to affect haemoglobin balance also results in increased deposition of fat in the liver. The fat content of the liver increased from 3.7 % in Group I to 7.0 % in Group III, which is significant. While the fat content rose the protein content fell significantly from 11 % in Group I to 9 % in Group III. In Group IV these changes in the composition of the liver were still more pronounced. On the other hand the liver protein and liver fat were the same in Group II as in Group I. Haemoglobin formation and the composition of the liver would thus appear to be influenced by protein deficiency at about the same dietary protein level, somewhere between 9 % and 4.5 % of protein. This, of course, has only been shown for the conditions obtaining in this experiment: selection of animals, composition of the diet, duration of the experiment, etc.

Some idea of the effect of protein deficiency on an organ can be obtained by examining its weight in relation to diminishing protein

intake. In general a fall in weight occurs if the protein content of the diet is inadequate, but in this experiment the fatty livers in Groups III and IV weighed at least as much as the livers in Groups I and II. The weights of the livers in the four groups were 16.66, 14.76, 17.25, and 16.32 g respectively (see Table 7 b): the mean weight of the liver was thus 2.5 g higher in Group III than in Group II, the difference being probably significant. The kidneys were of approximately the same weight in Groups I and II, whereas in Group III they weighed 22 % less than in Group I, the difference being significant. The kidneys in Group IV were 39 % lighter in weight than those in Group I. The spleen, too, was of the same weight in Groups I and II, but weighed 17 % less in Group III than in Group I (this difference, however, is not significant). In Group IV the spleen weighed 29 % less than in Group I, and here the difference is significant. Two endocrine organs were also weighed, viz. the testes and the adrenals. The testes showed only small weight differences in the first three groups, and had approximately the same weight in Group III as in Group I; in Group IV the weight of the testes was 31 % lower than in Group I, a probably significant difference. The adrenals, in contrast to the testes, diminished greatly in weight in Group III, in which they weighed 30.3 mg compared with 41.4 mg in Group I (a significant difference). In Group IV only two adrenal weights were recorded, this determination not having been performed in the first animals examined.

Like the total haemoglobin, the liver protein is placed in relation to the protein of the carcase; similarly the weights of other internal organs for which details of protein content are lacking. The quotient mg liver protein per g carcase protein reveals no significant differences between the different groups, the mean of the quotient being 43 in Group I, 38 in Group II, 40 in Group III, and 39 in Group IV. The kidneys diminish more in weight during protein deficiency than the carcase protein: the mean quotients mg kidney per g carcase protein are 90 in Group I, 90 in Group II, 79 in Group III, and 72 in Group IV. If Groups I and II are considered as a single group, and III and IV as a single group, the difference between the two mean quotients is highly significant. The decrease in weight of the kidneys during protein deficiency exhibits certain similarities to the fall in total haemoglobin: firstly, the kidneys begin to show a fall in weight in Group III and, secondly, their weight diminishes more than the carcase protein when the protein content of the diet is low enough. The mean quotients mg spleen per g carcase protein are 21, 22, 20, and 21, for each of the groups respectively, i.e. approximately the same in all cases. The quotients mg testes per g carcase protein are 55 in Group I, 60 in Group II, 57 in Group III, and 50 in Group IV: the difference between Group IV and the other groups combined is not significant. In the case of the adrenals the quotient mg adrenal per g carcase protein is found to

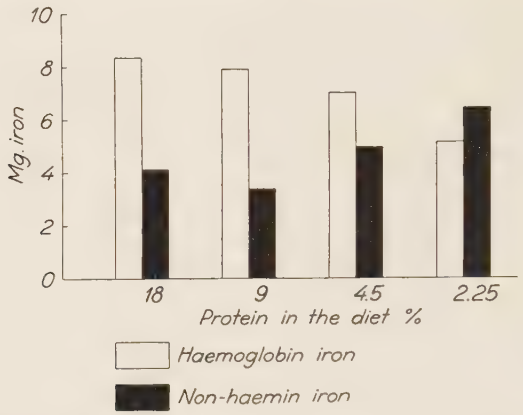

Fig. 5. Haemoglobin iron and non-haemin iron in adult rats on different protein intakes.

have fallen to 0.78 in Group III, compared with 0.95 in Group I and 0.94 in Group II. The difference between Group III and the combined Groups I and II is probably significant ($P < 0.02$): if the quotients for the two adrenal weights available in Group IV are added to Group III the difference between the low-protein group and the other animals becomes significant.

Since the diet in Group IV was so low in protein, it might be expected that the loss in weight should have taken place at the expense of the body protein in particular. The proportion of protein in the carcase, however, as can be seen from Table 7 b, was just as high in this group as in the others. The differences between the means for the various groups were also small in the case of the proportion of salts + carbohydrates, water, and fat, in the carcase. Since the proportion of salt was not raised as the result of the weight-loss in Group IV it must be supposed that the animals lost a certain amount of salts from the skeleton.

Simultaneously with the fall in total haemoglobin in Groups III and IV there was an increase in storage iron in the animals of these groups (see Table 7 a and Fig. 5). It is assumed in the following discussion that the animals in all groups had about the same amount of storage iron at the start of the experiment as those in Group I. The storage iron changes are stated in relation to the storage iron in Group I: in Group III the liver was responsible for nearly 80 % of the increase in storage iron. The liver would thus seem to be the organ in which is deposited the greatest fraction of the surplus iron resulting from a fall in the total haemoglobin. The storage iron in both liver and spleen increased sharply in Group IV; the non-haemin iron in the carcase

was also raised, but not significantly. In this group the liver and spleen were responsible for 72 % of the increase in storage iron in relation to Group I. By calculating the amount of iron representing the differences in total haemoglobin between Groups III and I and Groups IV and I, it is found that approximately 60 % was recovered as storage iron in Group III and about 70 % in Group IV. Such calculations are, of course, only approximate, because the storage iron in Groups III and IV at the start of the experiment probably differed more or less from the mean value in Group I. The storage iron in liver, spleen, and carcase was lower in Group II than in Group I, but the differences are not significant.

The variations in plasma iron within the groups were so great that it is impossible to say whether any differences existed between the groups. Finally, on consideration of the correlation between plasma iron and storage iron, it is found that each seemed to vary independently of the other within the limits of the variations in this experiment.

Discussion

Since the diet used was so poor in iron, and the animals did not receive any extra iron, the first point for discussion must be whether iron was not a limiting factor for haemoglobin formation. In the first place it must be established whether the animals had any storage iron which could be utilized for haemoglobin synthesis. In this connection I must anticipate the results of the iron-deficiency experiments I have done in growing rats. These establish that when the iron stores are exhausted the liver contains not more than 0.9–1.0 mg, the spleen less than 4–5 mg, and the carcase less than 0.5–0.6 mg, of non-haemin iron per 100 g fresh tissue. It is obvious that the animals in Groups III and IV had considerable stores of iron in liver, spleen, and carcase. On the other hand, several of the animals in Groups I and II had practically no storage iron in the liver (Group I, animals 1, 4, and 5; Group II, animals 7, 10, and 11): in these animals the spleen's content of iron was also low, but there was a certain amount of storage iron in the carcase. From this it follows that the liver and spleen contain iron stores which are easily mobilized at need. Since large quantities of storage iron could still be recovered from the carcase it is improbable that the iron intake on a diet otherwise balanced had a limiting effect upon haemoglobin formation. As a control, however, the haemoglobin concentration was estimated in 6 stock males on stock diet and of the same weight and age as the animals in Group I: the average haemoglobin concentration was 15.9 g per 100 ml (15.2–16.9), and the average total haemoglobin 2.52 g (2.45–2.57). These figures and the figures for the males in Group I (Hb. concentration 15.2 g per 100 ml, and

total haemoglobin 2.45 g) must be said to conform well, especially since the animals of the experiment lived in small cages and the stock animals were able to run about more freely in larger cages. It should be added that the three fattest animals in Groups I and II were the ones with the lowest haemoglobin concentration.

Concerning the percentage composition of the liver two points should be stressed. Firstly, all the blood has been washed out of the liver, and is replaced by perfusion fluid: this results in a lowering of the percentage content of protein in the liver, and an increase in the proportion of water. Secondly, it cannot be excluded that a surplus of perfusion fluid has remained in the liver. The protein level usually reported for the normal rat liver—about 18 % (Greenstein, 1947; and others)—is higher than that found for the viviperfused liver in this experiment (11 % in Group I). Here, however, it is not so much the absolute values but rather the comparative values of the different groups which are of interest. The above remarks concerning the liver apply also to other viviperfused organs and to the viviperfused carcase: in the case of the carcase, dry-weight determinations have shown that the increase in water-content due to the viviperfusion is less than 5 % of the carcase weight.

The animals in Group III consumed about 39 mg of nitrogen per 100 g of body-weight per day, and during an experimental period of 93 days were able to maintain their body-weight. Subsequent experiments have shown that this nitrogen intake approximately corresponds to the minimum nitrogen requirements for maintaining the body-weight in the adult rat when the composition of the diet is as used in this experiment. Goettsch (1951) found that the adult rat requires about 53 mg of nitrogen per 100 g body-weight per day to maintain its weight for 28 days: the protein in the diet consisted of a mixture of polished rice, 54.6 parts; red kidney beans, 28.2 parts; and crude casein, 15.4 parts. According to Goettsch the protein requirement for maintenance of the body-weight in the adult rat is 1.6 times greater than that for maintenance of nitrogen equilibrium. Since I have used protein of such high biological value as spray-dried hen's egg-white, and a longer experimental period, my figures and Goettsch's for the minimum requirement of nitrogen for maintaining the body-weight in the adult rat are in concordance.

Summing up the results of these experiments on haemoglobin formation in protein deficiency it may be said that haemoglobin formation has no particularly privileged position in relation to other demands in the competition for material for protein synthesis—in any event this is so in the adult male rat on a protein-deficient diet, and not subjected to blood-letting. Haemoglobin formation begins to be affected at a stage when the animal is still able to adapt itself to a low protein intake so as to achieve positive nitrogen balance and maintain body-

weight. When the protein content of the diet has had a limiting effect on the synthesis of protein in the organism to the extent that the total protein in the carcases of young adult male rats is, after a period of 2–4 months, *c.* 10 % lower than in controls on a high-protein diet, the haemoglobin formation has been limited to at least the same, and probably somewhat greater, extent. The fall in total haemoglobin appears simultaneously with several other signs of protein deficiency. Thus there is increased deposition of fat in the liver and a fall in the liver protein, and the kidneys and adrenals diminish in weight.

When the protein content of the diet is reduced to 2.25 % of spray-dried hen's egg-white, the total haemoglobin falls more than the carcase protein.

It would seem reasonable to suppose that that protein in the body which is rapidly metabolized should be affected more by inadequate protein intake than the protein which is replaced more slowly. This would, of course, be compensated by a high degree of priority for the building up of the protein in question. The degree of priority of haemoglobin formation in adult male rats does not, however, appear to be higher than that the quotient g Hb. per g body protein falls if the dietary protein becomes too low.

The pronounced fall in adrenal weight which accompanies the diminution of the total haemoglobin on reducing the dietary protein seems to me to be of particular interest. The adrenals behave differently from the testes (the other endocrine organ weighed), and the fall in weight which they exhibit may well be interpreted as an expression of a regulatory mechanism by which the organism adapts itself to a lower protein intake. In the experiments which follow, further attention will be directed towards the weight of the adrenals in protein deficiency.

CHAPTER IV A

Dietary Factors in the Aetiology of Idiopathic Hypochromic Anaemia

Discussion

It is not yet clear how the idiopathic hypochromic anaemia develops. Since it can be cured simply by the administration of iron it would seem obvious to suppose low dietary iron to be the primary aetiological factor. The human requirement of iron under different physiological conditions is among the less perfectly understood problems of nutrition. To get some idea of the normal requirement of iron, both iron-balance experiments and determinations of the iron content of the diet of population groups with free choice of food have been done. Iron-balance experiments, however, present many technical difficulties: iron in different compounds is utilized to different extents, and other constituents of the diet may influence absorption of the iron. Sherman (see Sherman, 1946) came to the conclusion, from the results of his own iron-balance experiments and those of several other researchers, that the smallest amount of dietary iron with which equilibrium can be maintained amounts to slightly less than 8 mg per day. To this 8 mg he adds a safety margin of 50 % to cover individual variations, making the daily requirement 12 mg of iron. The American National Research Council on Food and Nutrition also recommends a minimum daily intake for adults of 12 mg of iron, the corresponding figure for pregnancy and lactation being 15 mg.

The iron requirement, however, would appear to be to some extent dependent on the composition of the diet, and to be considerably below these figures when the diet is balanced. Leverton (1941) found the iron balance to be negative in four healthy women undergraduates on an adequate diet giving 3.5–4.5 mg of iron per day, and that when the daily iron intake was raised to 6.55 mg they retained on an average 2.14 mg of iron per day. Leverton and Marsh (1942) found that seven young women receiving an average of 7.21 mg of iron per day and a diet adequate in other respects retained 1.55 mg of iron per day, i.e. more than enough to cover the menstrual loss. Five other women with similar iron intake (7.00 mg of iron per day), but on a diet suboptimal

in other respects (calcium, phosphorus, protein), had, in contrast, negative iron balance (−1.30 mg iron). The British Medical Research Council's Committee on Haemoglobin Surveys (1945) estimated the minimum requirement of iron to be 5–6 mg per day, provided that "none of the factors causing an increased demand for iron is operative". Whipple suggests that as little as 2–3 mg suffices for the adult. Johnston and McMillan (1952) have published an investigation according to which 6.42 mg of iron per day is inadequate for women. Six young women undergraduates lived on a carefully controlled diet during 12 weeks. On an average daily iron intake of 6.42 mg three of the women retained insufficient iron to cover their menstrual loss and at the same time provide a small margin of safety for other losses (hair, shed epithelial cells, sweat, etc.). When the dietary iron was raised to 11.52 mg per day all of them retained during 8 weeks sufficient iron from the diet to cover their losses.

Since estimations of the human requirement of iron are so uncertain, and the requirement appears to vary according to the composition of the diet, it is obviously no easy matter to determine whether the daily iron intake has been too low in population groups in which hypochromic anaemia is common. Odin (1934) found a high frequency of idiopathic hypochromic anaemia in the coastal districts of Västerbotten (Northern Sweden), especially in women. Iron analyses of the types of foodstuffs commonly in use, and the weighing of all food consumed in a few individual cases, revealed that the average daily iron intake in the case of the women amounted to 11 mg (8.4–12.3). Although the iron content of the diet was thus relatively low, it would hardly appear low enough to be the sole explanation of the frequent incidence of idiopathic hypochromic anaemia in that district. In fact, Widdowson and McCance (1936) found the average daily iron intake of English middle-class women on freely chosen diets to be 11.4 mg. Lundholm (1939) estimated the average daily iron consumption in 29 cases of idiopathic hypochromic anaemia from Jämtland (a province in Northern Sweden) to be 9.6 mg ($\sigma = \pm 3.2$): in some of these cases the iron intake was thus undoubtedly low enough to explain the iron deficiency. He was, however, unable to correlate the degree of anaemia and the intake of iron. Davidson and Fullerton (1933) estimated the dietary iron of 49 poor families in Aberdeen. Twenty-five of the housewives had hypochromic anaemia, twenty-four had not. There was no difference in iron consumption between the families in which there were cases of anaemia and those in which there were not. The men consumed on an average 10.71 mg of iron per day. If the women's consumption of food is taken to be 80 % of the men's, their intake of iron should be 8.57 mg per day. Fullerton later (1936) corrected these figures; 10 % of the iron was found to be lost during cooking, and the iron content of "golden syrup", consumed in large quantities by poor families in Aberdeen, was found to be lower

than previously stated. He then concluded that the women consumed, on an average, 7.3 mg of iron per day. Waldenström (1946) found idiopathic hypochromic anaemia to be common in the country districts of Upland and Gotland (provinces in mid-Sweden). Blix (1947) was unable to demonstrate any difference in the iron content of common foodstuffs from farms with and without cases of anaemia in certain of the parishes of Upland and Gotland touched by Waldenström's investigations.

In summary, iron intake seems to have been relatively low in population groups with a high frequency of idiopathic hypochromic anaemia, but in many cases of anaemia it must have been enough to cover a normal requirement of iron, provided the diet was otherwise adequate. It is obvious, however, that the population groups in question lived on a diet which, besides a relative iron deficiency, was also characterized by a deficiency of protein (Odin, 1934; Fullerton, 1936; Kennedy, 1939; Mackay, Wills, Dobbs, and Bingham, 1942). The intake of animal protein in particular was low, and the diet also appears to have been deficient in vitamins. Odin found, for example, that the women in Västerbotten, who lived on a diet consisting exclusively of milk and cereals, consumed no more than 42–61 g of protein per day. On the basis of such findings one is tempted to believe that low dietary iron is particularly liable to cause idiopathic hypochromic anaemia when the diet is also deficient in protein. This could be explained by assuming the absorption of iron to be less, or the iron losses to be greater, when the diet is low in protein. A further possibility, already suggested in the Introduction, might be as follows. When material for protein synthesis is scanty, and there is competition for it, it is possible that the position of haemoglobin formation changes according to the amount of storage iron available: a rich supply of iron in the stores would stimulate haemoglobin formation by exerting some kind of mass action. According to this theory haemoglobin formation would be particularly impeded by a low protein intake when the supply of storage iron is small. Thus, I considered it desirable to study haemoglobin formation in rats on varying the intake of both iron and protein. I have not been able to find descriptions of any such experiments in the literature, in which haemoglobin formation during simultaneous deficiency of protein and iron has been studied.

CHAPTER IV B

Haemoglobin Formation, Plasma Iron, and Storage Iron, in Growing Rats on Varying Intake of Iron and Protein

Adult animals should not be used in studying haemoglobin formation during simultaneous deficiency of iron and protein in rats not subjected to blood-letting: protein deficiency causes in these a fall in the total haemoglobin, the iron liberated from the haemoglobin being laid down in the stores. Further, adult animals are less dependent upon dietary iron than growing animals, which have to increase their blood volume. In these double-deficiency experiments I have therefore used young growing animals.

Experimental

Sixty rats aged 25–28 days and weighing 39–67 g were divided into two equal groups. The first (Group A) were kept on a full protein diet containing 22 % of spray-dried hen's egg-white, and the second (Group B) on a low-protein diet starting with 9 % of spray-dried hen's egg-white. As the animals became bigger they grew better on a diet containing only 9 % of protein; therefore, in order to distinguish more clearly between the growth rates of the low-protein animals and the 22 % animals, the dietary protein of the former group was reduced after 45 days to 8 %, after 68 days to 4.5 %, and after 82 days to 4 %. Both of the groups were divided into 5 sub-groups (A I–A V and B I–B V), with 6 rats to each group. The animals of each of the ten sub-groups had approximately the same mean weight, and the various litters were equally divided between the sub-groups: the first five animals in each group were males and the sixth a female. The animals were placed in individual cages of chromium-plated brass netting.

Both the diet containing 22 % protein and the low-protein diet were extremely deficient in iron (only 2.5 µg of iron per g of diet). The different sub-groups were given various supplements of iron as ferrous sulphate: A I and B I received 560 µg, A II and B II 280 µg, A III and B III 70 µg, and A IV and B IV 35 µg of iron per day (A V and B V received no supplement). The ferrous sulphate was mixed with

lactose so that 100 mg of the mixture contained a day's dose of iron. The different mixtures were weighed into gelatin capsules (100 mg in each), and the content of one capsule mixed with the daily ration of feed. The composition of the diet was otherwise the same as that previously described (see Methods, p. 95). The amount of cane sugar varied from 69 to 87 % according to the proportion of protein in the diet.

The animals were given each day a weighed quantity of feed resembling as closely as possible their *ad lib.* consumption. Since the daily dose of iron was mixed with the feed it was important not to give more feed than they could consume. For the first three days all the animals received 5 g of feed. This was plainly too little, and so the ration was first increased to 9 g and after a week to 10 g. At 18 days the difference in body-weight between Group A and Group B was so great that they could no longer be kept on the same amount of feed, and this had to be adjusted after the body-weight: when the animals reached 100 g in weight they were given 12 g of feed, and when they reached 150 g the daily ration was increased to 14 g, on which latter amount they were kept for the remainder of the period. In addition to the iron supplements all animals thus consumed 25–35 µg of iron per day owing to the iron content of the diet.

The animals were weighed, as a rule, once a week during the course of the experiment. Each animal was kept on experiment until it reached about 200 g in weight. The experimental period was thus considerably longer for the low-protein animals.

At the end of the experiment the following determinations were done on each animal: haemoglobin concentration, red cell count, haematocrit reading, total haemoglobin, total amount of protein in the carcase, plasma iron, and non-haemin iron in the liver, spleen, and carcase. In addition some of the organs were weighed, among them the adrenals.

Results and Discussion

The animals on 22 % dietary protein consumed completely the feed supplied each day, as did also the low-protein animals with the exception of one or two during the first days of the experiment. The rate of growth was quite independent of the iron intake, and in the growth curves, Fig. 6, all animals on 22 % dietary protein are therefore treated as a single group and the low-protein animals treated as a second group. The growth curves end at the point where the first animals in each group were killed.

All results concerning the 22 % animals are collected in Tables 8 a and 8 b, and those concerning the low-protein animals in Tables 9 a and 9 b. In Tables 8 a and 9 a can be found the data on haemoglobin,

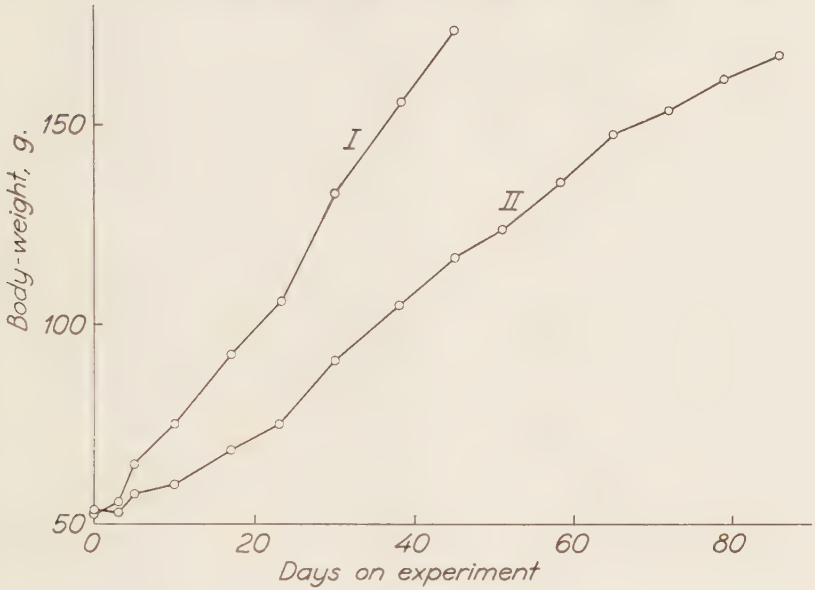

Fig. 6. Growth curves of rats on a diet with 22 % protein and on a low-protein diet.

red blood cells, and iron metabolism; details of body-weights and organ-weights, total carcase protein, and the percentage content of protein and salts + carbohydrates in the carcase, are collected in Tables 8 b and 9 b.

Protein Deficiency in Growing Rats

The groups of animals on 280 and 560 µg of iron per day received iron in excess of the minimum requirement of the growing rat. The low-protein animals on these doses thus constitute a pure protein-deficiency experiment on growing rats. I considered it particularly important that the low-protein animals in these groups should, at the end of the experiment, resemble in weight as closely as possible the corresponding 22 % animals. By this means the study of the total haemoglobin as a function of the protein content of the diet is simplified.

The 22 % animals had very similar blood pictures in the groups receiving 280 and 560 µg of iron per day. It was in any case impossible to raise the haemoglobin concentration or the total haemoglobin by increasing the dose of iron. Nor in the low-protein animals were there any significant differences in haemoglobin concentration, red cell count,

Table 8 a. Haemoglobin, red blood cells, non-haemin iron, and plasma iron, in growing rats on diets containing 22 % protein and different amounts of iron.

Rat No.	Hb. concentration, g per 100 ml	Red blood cells, millions per mm³	Volume of packed red cells, ml per 100 ml	Mean corpuscular volume, cu. microns	Mean corpuscular haemoglobin, micromicrograms	Mean corpuscular Hb. concentration, %	Total haemoglobin, g	Plasma iron, µg per 100 ml	Non-haemin iron in liver, mg	Non-haemin iron in spleen, mg	Non-haemin iron in carcase, mg	Total non-haemin iron, mg
									Figures in brackets indicate mg non-haemin iron per 100 g fresh tissue			
Group A I. 560 µg iron per day.												
1	18.1	7.6	46	61	24	39	1.82	226	0.362 (3.35)	0.172 (39.05)	1.84 (1.12)	2.37
2	15.8	6.6	45	68	24	35	1.36	285	0.378 (3.24)	0.084 (26.38)	1.38 (1.05)	1.84
3	15.9	6.2	47	76	26	34	1.37	311	0.356 (3.26)	0.077 (18.84)	1.47 (1.07)	1.90
4	12.5	5.6	43	77	22	29	1.30	135	0.295 (2.93)	0.047 (12.81)	1.69 (1.36)	2.03
5	17.1	7.6	48	63	23	36	1.86	189	0.352 (3.07)	0.077 (15.98)	1.40 (0.85)	1.83
6	17.0	6.6	44	67	26	39	1.52	239	0.902 (12.13)	0.525 (128.05)	1.84 (1.28)	3.27
Mean	16.1	6.7	46	69	24	35	1.54	231	0.441 (4.66)	0.164 (40.19)	1.60 (1.12)	2.21
Group A II. 280 µg iron per day.												
7	16.5	6.6	44	67	25	38	1.36	217	0.342 (3.41)	0.033 (5.50)	1.28 (0.96)	1.66
8	17.6	6.8	48	71	26	37	2.05	230	0.374 (3.62)	0.104 (16.30)	1.41 (0.83)	1.89
9	16.8	6.2	49	79	27	34	1.63	207	0.178 (1.43)	0.028 (4.43)	1.27 (0.94)	1.48
10	15.1	6.1	43	70	25	35	—	175	0.363 (3.80)	0.157 (12.34)	1.50 (1.07)	2.02
11	15.9	6.7	45	67	24	35	1.31	143	0.175 (1.70)	0.066 (6.07)	1.23 (0.92)	1.47
12	18.4	7.5	46	61	25	40	1.43	232	0.781 (8.54)	0.353 (59.85)	1.69 (1.25)	2.82
Mean	16.7	6.7	46	69	25	37	1.56	201	0.369 (3.75)	0.124 (17.42)	1.40 (1.00)	1.89

Group A III. 70 μg iron per day.

13	10.3	6.6	32	48	16	32	1.06	35	0.070 (0.64)	0.012 (2.02)	0.90 (0.54)	0.98
14	10.8	6.7	32	48	16	34	1.05	47	0.106 (1.00)	0.015 (4.24)	0.88 (0.56)	1.00
15	12.2	7.4	43	58	16	28	1.11	34	0.065 (0.70)	0.011 (2.44)	0.84 (0.53)	0.92
16	13.3	6.8	40	59	20	33	1.36	87	0.079 (0.82)	0.013 (3.21)	0.96 (0.57)	1.05
17	13.4	6.5	43	66	21	31	1.21	73	0.091 (0.92)	0.016 (3.12)	0.94 (0.52)	1.05
18	14.8	7.4	42	57	20	35	1.42	84	0.105 (1.35)	0.021 (3.80)	0.80 (0.51)	0.93
Mean	12.5	6.9	39	56	18	32	1.20	60	0.086 (0.91)	0.015 (3.14)	0.89 (0.54)	0.99

Group A IV. 35 μg iron per day.

19	9.5	6.3	27	43	15	35	1.01	32	0.055 (0.49)	0.009 (1.16)	0.62 (0.35)	0.68
20	7.8	7.0	26	37	11	30	0.79	48	0.114 (0.99)	0.015 (2.54)	1.06 (0.60)	1.19
21	8.3	6.2	28	45	13	30	0.72	28	0.109 (1.04)	0.019 (3.06)	0.79 (0.50)	0.92
22	6.7	6.5	23	35	10	29	0.81	33	0.090 (0.93)	0.016 (1.70)	0.63 (0.36)	0.74
23	8.4	7.5	27	36	11	31	0.74	37	0.076 (0.79)	0.013 (1.53)	0.71 (0.41)	0.80
24	9.9	7.6	32	42	13	31	0.80	66	0.096 (1.25)	0.024 (4.03)	0.70 (0.50)	0.82
Mean	8.4	6.9	27	40	12	31	0.81	41	0.090 (0.92)	0.016 (2.34)	0.75 (0.45)	0.86

Group A V. 0 μg iron per day.

25	6.4	6.2	20	32	10	32	0.72	34	0.089 (0.90)	0.017 (3.39)	0.56 (0.31)	0.67
26	5.7	4.7	21	45	12	27	0.38	22	0.111 (1.15)	0.015 (3.21)	0.74 (0.48)	0.87
27	6.8	6.2	24	39	11	28	0.74	23	0.103 (0.90)	0.015 (3.59)	0.82 (0.49)	0.94
28	6.2	6.2	19	31	10	33	0.67	24	0.089 (0.85)	0.015 (2.29)	0.80 (0.46)	0.90
29	4.3	5.0	17	34	9	25	0.54	33	0.073 (0.76)	0.011 (1.25)	0.87 (0.50)	0.95
30	6.8	6.7	24	36	10	28	0.51	30	0.071 (1.03)	0.010 (2.39)	0.59 (0.45)	0.67
Mean	6.0	5.8	21	36	10	29	0.59	28	0.089 (0.93)	0.014 (2.69)	0.73 (0.45)	0.83

Table 8 b. Body-weight, weight of organs, percentage of protein and salts in the carcase, and total carcase protein, in growing rats on a diet containing 22 % protein and different amounts of iron.

Rat No.	Days on experiment	Body-weight at beginning of experiment g	Body-weight at end of experiment g	Weight of carcase g	Protein in carcase %	Salts and carbohydrates in carcase %	Weight of liver g	Weight of spleen g	Weight of adrenals mg	Total amount of protein in the carcase g	g Hb. per g protein in the carcase
Group A I. 560 µg iron per day.											
1	75	42	217	164	16.9	7.0	10.81	0.44	31.7	27.7	0.066
2	50	42	185	131	17.4	6.1	11.68	0.32	33.4	22.8	0.060
3	46	52	188	137	15.2	6.0	10.91	0.41	35.8	20.8	0.066
4	41	60	173	124	17.3	6.6	10.08	0.37	27.9	21.4	0.061
5	61	64	220	165	15.7	5.8	11.48	0.48	37.7	25.9	0.072
6	81	50	193	144	15.3	6.6	7.44	0.41	38.6	22.0	0.069
Mean	59	52	196	144	16.3	6.4	10.40	0.41	34.2	23.4	0.066
Group A II. 280 µg iron per day.											
7	48	44	190	133	16.3	6.6	10.04	0.60	33.4	21.7	0.063
8	72	44	235	170	17.1	6.3	10.34	0.64	39.1	29.0	0.071
9	45	52	199	135	16.8	6.4	12.44	0.63	40.6	22.7	0.072
10	42	61	204	140	17.1	6.3	9.55	1.27	32.4	23.9	—
11	47	63	181	134	16.3	6.0	10.31	1.08	31.3	21.8	0.060
12	86	56	191	135	16.4	6.9	9.15	0.59	38.3	22.2	0.064
Mean	57	53	200	141	16.7	6.4	10.31	0.80	35.9	23.6	0.066

Group A III. 70 μg iron per day.

13	58	48	219	166	16.0	6.0	10.94	0.57	38.5	26.5	0.040
14	55	44	215	158	17.1	5.9	10.57	0.36	32.8	27.0	0.039
15	53	50	213	159	15.7	6.0	9.32	0.44	41.7	25.0	0.044
16	60	64	220	168	15.8	6.1	9.59	0.41	32.7	26.5	0.051
17	67	64	238	180	15.9	6.2	9.92	0.51	33.6	28.6	0.042
18	73	55	200	157	14.3	5.9	7.77	0.56	39.8	22.5	0.063
Mean	61	54	218	165	15.8	6.0	9.69	0.48	36.5	26.0	0.047

Group A IV. 35 μg iron per day.

19	62	47	230	176	15.5	6.2	11.13	0.80	31.7	27.3	0.037
20	66	44	241	177	16.3	5.5	11.49	0.61	34.3	28.8	0.027
21	54	52	206	157	15.5	5.6	10.49	0.62	32.5	24.3	0.030
22	69	56	229	176	14.6	5.1	9.63	0.94	29.2	25.7	0.032
23	70	52	225	172	14.8	5.8	9.67	0.83	29.8	25.5	0.029
24	82	54	189	139	17.1	6.2	7.67	0.60	47.8	23.7	0.034
Mean	67	51	220	166	15.6	5.7	10.01	0.73	34.2	25.9	0.032

Group A V. 0 μg iron per day.

25	68	50	232	181	16.4	5.8	9.92	0.49	32.3	29.6	0.024
26	52	42	200	154	16.5	5.7	9.69	0.48	33.2	25.4	0.015
27	59	52	232	168	16.8	6.5	11.45	0.41	35.9	28.2	0.026
28	74	60	235	173	16.5	6.4	10.45	0.67	38.0	28.6	0.023
29	64	54	230	173	14.8	5.7	9.59	0.85	42.2	25.6	0.021
30	83	52	178	131	17.4	6.7	6.89	0.42	38.1	22.8	0.022
Mean	67	52	218	163	16.4	6.1	9.67	0.55	36.6	26.7	0.022

Table 9 a. Haemoglobin, red blood cells, non-haemin iron, and plasma iron, in growing rats on a low-protein diet containing different amounts of iron.

Rat No.	Hb. concentration, g per 100 ml	Red blood cells, millions per mm³	Volume of packed red cells, ml per 100 ml	Mean corpuscular volume, cu. microns	Mean corpuscular haemoglobin, micromicrograms	Mean corpuscular Hb. concentration, %	Total haemoglobin, g	Plasma iron, µg per 100 ml	Non-haemin iron in liver, mg	Non-haemin iron in spleen, mg	Non-haemin iron in carcase, mg	Total non-haemin iron, mg
									Figures in brackets indicate mg non-haemin iron per 100 g fresh tissue			
Group B I. 560 µg iron per day.												
1	14.8	8.3	39	47	18	38	1.11	124	0.794 (9.72)	0.134 (58.26)	1.17 (0.82)	2.10
2	13.0	7.8	37	47	17	35	1.03	75	1.491 (16.98)	0.109 (45.43)	0.94 (0.63)	2.54
3	14.7	7.4	42	57	20	35	1.40	110	0.985 (10.64)	0.283 (41.00)	1.25 (0.84)	2.52
4	15.5	7.5	41	55	21	38	1.25	155	1.618 (19.95)	0.265 (42.81)	1.54 (1.15)	3.42
5	15.6	(5.3)	41	—	—	38	1.23	199	0.708 (8.79)	0.206 (45.67)	1.50 (1.08)	2.41
6	16.7	7.7	47	61	22	36	1.15	187	0.756 (10.55)	0.165 (63.60)	1.32 (0.94)	2.24
Mean	15.1	7.7	41	53	20	37	1.20	142	1.059 (12.77)	0.194 (49.46)	1.29 (0.91)	2.54
Group B II. 280 µg iron per day.												
7	15.3	7.8	45	58	20	34	1.25	134	0.735 (7.90)	0.116 (24.58)	1.20 (0.83)	2.05
8	14.1	7.3	40	55	19	35	0.90	100	0.065 (0.80)	0.018 (4.39)	1.05 (0.83)	1.13
9	13.8	7.8	42	54	18	33	1.22	140	0.251 (2.80)	0.073 (11.58)	1.06 (0.83)	1.38
10	15.5	(5.9)	44	—	—	35	1.37	155	0.432 (4.32)	0.088 (17.20)	1.43 (1.03)	1.95
11	13.2	7.6	41	54	17	32	1.11	104	0.215 (3.21)	0.025 (4.69)	1.14 (0.80)	1.38
12	14.1	7.4	45	61	19	31	1.11	193	0.620 (7.19)	0.132 (39.85)	1.56 (1.15)	2.31
Mean	14.3	7.6	43	56	19	33	1.16	138	0.386 (4.37)	0.075 (17.05)	1.24 (0.91)	1.70

Group B III. 70 μg iron per day.

13	13.2	7.7	40	52	17	33	1.00	115	0.085 (0.88)	0.016 (5.97)	0.98 (0.69)	1.08
14	13.8	7.1	41	58	19	34	1.23	116	0.113 (1.00)	0.012 (3.31)	1.10 (0.74)	1.23
15	11.9	7.8	38	49	15	31	1.19	94	0.099 (1.05)	0.012 (4.01)	0.94 (0.60)	1.05
16	13.3	7.5	36	48	18	37	1.20	79	0.047 (0.63)	0.019 (4.30)	0.93 (0.65)	1.00
17	14.2	7.4	42	57	19	34	1.22	124	0.161 (1.82)	0.029 (9.52)	1.22 (0.89)	1.41
18	14.4	8.0	42	53	18	34	1.06	138	0.096 (1.24)	0.029 (9.86)	1.04 (0.72)	1.17
Mean	13.5	7.6	40	53	18	34	1.15	111	0.100 (1.10)	0.020 (6.16)	1.04 (0.72)	1.16

Group B IV. 35 μg iron per day.

19	11.5	7.7	40	52	15	29	0.94	53	0.030 (0.30)	0.009 (2.20)	0.70 (0.48)	0.74
20	10.3	7.1	41	58	15	25	0.87	55	0.067 (0.85)	0.012 (4.59)	0.70 (0.55)	0.78
21	14.4	7.8	38	49	18	38	1.18	86	0.068 (0.74)	0.014 (3.38)	0.80 (0.52)	0.88
22	10.2	7.5	36	48	14	28	0.94	61	0.059 (0.76)	0.016 (3.32)	0.60 (0.42)	0.68
23	9.6	7.4	42	57	13	23	1.05	49	0.066 (0.84)	0.016 (2.78)	0.73 (0.49)	0.81
24	11.0	8.0	42	53	14	26	0.84	70	0.066 (0.75)	0.008 (3.07)	0.63 (0.47)	0.70
Mean	11.2	7.6	40	53	15	28	0.97	62	0.059 (0.71)	0.013 (3.22)	0.69 (0.49)	0.77

Group B V. 0 μg iron per day.

25	6.0	7.0	21	30	9	29	0.41	—	0.054 (0.56)	0.013 (2.78)	0.49 (0.32)	0.56
26	7.7	7.5	24	32	10	32	0.51	38	0.065 (0.71)	0.012 (3.12)	0.54 (0.38)	0.62
27	6.9	7.1	22	31	10	31	0.62	32	0.045 (0.49)	0.010 (2.80)	0.59 (0.44)	0.65
28	7.5	7.9	28	35	9	27	0.65	34	0.056 (0.73)	0.003 (1.18)	0.55 (0.36)	0.61
29	8.5	7.8	27	35	11	31	0.74	31	0.059 (0.58)	0.018 (3.21)	0.59 (0.38)	0.67
30	8.5	8.3	29	35	10	29	0.64	45	0.084 (1.14)	0.010 (2.98)	0.53 (0.39)	0.62
Mean	7.5	7.6	25	33	10	30	0.60	36	0.061 (0.70)	0.011 (2.68)	0.55 (0.38)	0.62

Table 9 b. Body-weight, weight of organs, percentage of protein and salts in the carcase, and total carcase protein, in growing rats on a low-protein diet containing different amounts of iron.

Rat No.	Days on experiment	Body-weight at beginning of experiment	Body-weight at end of experiment	Weight of carcase	Protein in carcase	Salts and carbohydrates in carcase	Weight of liver	Weight of spleen	Weight of adrenals	Total amount of protein in the carcase	g Hb. per g protein in the carcase
		g	g	g	%	%	g	g	mg	g	
		Group B I. 560 µg iron per day.									
1	137	41	188	143	15.0	7.5	8.17	0.23	23.8	21.4	0.052
2	125	39	193	149	15.8	6.5	8.78	0.24	25.8	23.6	0.044
3	130	61	198	149	15.1	7.1	9.26	0.69	22.0	22.5	0.062
4	88	65	178	134	17.3	6.6	8.11	0.62	24.9	23.2	0.054
5	80	67	182	139	15.9	6.5	8.06	0.45	23.0	22.1	0.056
6	115	46	183	140	16.6	6.8	7.17	0.26	32.2	23.2	0.050
Mean	113	53	187	142	16.0	6.8	8.26	0.42	25.3	22.7	0.053
		Group B II. 280 µg iron per day.									
7	122	44	187	145	15.7	6.8	9.31	0.47	22.3	22.7	0.055
8	146	40	167	126	18.8	6.6	8.15	0.41	23.1	23.7	0.038
9	139	62	180	128	16.9	7.7	8.96	0.63	21.8	21.6	0.056
10	130	65	191	139	17.2	8.0	9.99	0.51	20.5	23.9	0.057
11	143	64	186	143	16.2	6.4	6.70	0.54	20.5	23.2	0.048
12	110	48	187	136	16.2	7.0	8.63	0.33	37.7	22.0	0.050
Mean	132	54	183	136	16.8	7.1	8.62	0.48	24.3	22.9	0.051

Group B III. 70 μg iron per day.

13	109	48	188	142	17.4	6.8	9.70	0.26	25.2	24.7	0.040
14	124	45	198	149	14.2	9.0	11.30	0.36	23.7	21.1	0.058
15	133	60	203	156	15.8	7.4	9.44	0.29	26.4	24.6	0.048
16	111	66	184	143	16.3	6.3	7.46	0.44	22.7	23.3	0.052
17	90	66	182	137	16.1	7.3	8.82	0.30	23.2	22.1	0.055
18	117	48	184	145	13.7	7.2	7.77	0.29	25.7	19.9	0.053
Mean	114	56	190	145	15.6	7.3	9.08	0.32	24.5	22.6	0.051

Group B IV. 35 μg iron per day.

19	106	48	190	145	14.6	7.5	9.91	0.39	21.5	21.1	0.045
20	149	44	174	128	15.6	7.2	7.87	0.26	19.7	19.9	0.044
21	89	50	192	154	14.4	6.7	9.19	0.40	23.6	22.2	0.053
22	120	50	192	142	14.9	7.1	7.76	0.47	21.9	21.2	0.044
23	132	66	191	148	15.5	6.7	7.90	0.56	23.3	22.9	0.046
24	123	52	182	135	13.4	9.4	8.85	0.25	29.8	18.1	0.046
Mean	120	52	187	142	14.7	7.4	8.58	0.39	23.3	20.9	0.046

Group B V. 0 μg iron per day.

25	108	51	200	154	15.7	6.5	9.59	0.45	23.2	24.2	0.017
26	140	46	192	141	15.9	7.4	9.18	0.39	26.1	22.4	0.023
27	87	52	190	135	15.4	8.0	9.15	0.35	24.2	20.8	0.030
28	116	51	201	152	15.5	6.3	7.66	0.27	22.3	23.6	0.028
29	105	64	206	154	16.0	7.4	10.19	0.55	23.8	24.7	0.030
30	136	50	189	137	15.3	6.9	7.38	0.33	33.4	20.9	0.031
Mean	115	52	196	146	15.6	7.1	8.86	0.39	25.5	22.8	0.027

haematocrit reading or total haemoglobin between the groups on a daily iron dose of 280 and 560 μg. The mean corpuscular Hb. concentration was somewhat higher in the low-protein group receiving 560 μg of iron than in the group on 280 μg of iron (37 % and 33 % respectively), the difference being significant. The total haemoglobin values for both low-protein groups were closely similar—1.20 g in the group receiving 560 μg of iron, and 1.16 g in the 280 μg group. Thus, neither in the low-protein animals was any mass effect of the iron seen on increasing the daily dose from 280 to 560 μg.

Since there are no great differences between the groups receiving 560 and 280 μg of iron on the same protein intake it is possible to combine the *22* % animals on 560 and 280 μg of iron in one group, and the low-protein animals on these iron doses in a second group. By comparing these groups the effect of limited protein intake upon haemoglobin formation in growing animals can be studied. The relevant data are collected in Table 10.

The low-protein animals developed *a slight anaemia* with the haemoglobin concentration *c.* 10 % lower than in the *22* % animals. The haematocrit was also nearly 9 % lower[1], whereas the red cell count was higher (7.7 million compared with 6.7 million in the *22* % animals). The mean corpuscular volume was thus considerably diminished (*c.* 20 %) in the low-protein animals, i.e. the low-protein anaemia is

Table 10. Comparison between growing rats on a diet with 22 % protein and on a low-protein diet. Daily iron intake 280 or 560 μg.

	I Animals with 22 % protein in the diet	II Low-protein animals	D_{I-II}	P
Body-weight at end of experiment, g	198	185		
Protein in the carcase, g	23.5	22.8		
Total haemoglobin, g	1.55	1.18	0.37	<0.001
g Hb. per g protein in the carcase	0.066	0.052	0.014	<0.001
Hb. concentration, g per 100 ml	16.4	14.7	1.7	<0.01
Red blood cells, millions per mm^3	6.7	7.7	−1.0	<0.001
Haematocrit reading, %	46	42	4	<0.01
Mean corpuscular volume, cu. microns	69	55	14	<0.001
Mean corpuscular haemoglobin, micromicrograms	25	19	6	<0.001
Weight of adrenals, mg	35	24.8	10.2	<0.001
Plasma iron, μg per 100 ml	216	140	76	<0.001

[1] Per cent of the mean value for the corresponding *22* % animals.

plainly microcytic. The mean corpuscular haemoglobin was 24 % lower in the low-protein animals, but the mean corpuscular Hb. concentration was found to be normal, or, on the lower dose of iron (280 µg per day), possibly slightly subnormal. The low-protein anaemia is thus of microcytic, and normochromic or slightly hypochromic type.

While the haemoglobin concentration is influenced only comparatively slightly by protein deficiency, *the total haemoglobin diminishes greatly.* The body-weight of the low-protein animals was, at the end of the experiment, on an average 6.6 % lower than that of the 22 % animals, and the total carcase protein only about 3 % lower. The total haemoglobin was as much as 24 % lower in the low-protein animals, the quotient g Hb. per g carcase protein being diminished from 0.066 in the 22 % animals to 0.052 in the low-protein animals, a fall of 21 %.

The adrenals showed a marked fall in weight from 35 mg in the 22 % animals to 24.8 mg in the low-protein group. This fall of 30 % is much greater than can be explained by the difference in body-weight (6.6 %).

The percentage of protein in the carcase was almost the same for the low-protein animals and the animals on 22 % dietary protein (Tables 8 b and 9 b), the mean for all 22 % animals being 16.2 % and the mean for all low-protein animals 15.7 %—a difference which is not significant. Both in the low-protein animals and the 22 % animals the percentage of protein in the carcase was lowest in the group receiving 35 µg of iron per day. The difference between the low-protein group on 280 µg of iron per day with the highest mean value and the low-protein group on 35 µg of iron per day with the lowest mean value is significant. In the 22 % animals, too, the percentage of protein in the carcase was highest in the 280 µg group and lowest in the 35 µg group, the difference being probably significant. It is possible that the lower carcase protein in the anaemia groups on 35 µg of iron per day was due to the fact that the anaemic animals seemed to be comparatively fat, but the carcase fat was not estimated. The mean content of salts + carbohydrates in the carcase was for all low-protein animals 7.2 %, and for the 22 % animals 6.1 % (Tables 8 b and 9 b), the difference being highly significant.

As in the experiment on adult male rats, the analyses of the composition of the carcase done in this experiment prove that there is no great difference between animals on a low-protein diet and animals on an adequate protein intake. In particular, the percentages of protein in the carcases are very similar.

The low-protein animals in the groups on 280 and 560 µg of iron per day weighed about 7 % less than the 22 % animals in the corresponding groups; but the liver was as much as 20 % lighter. The quotient g liver per g carcase protein was significantly diminished in the low-protein animals: the liver protein, like the haemoglobin, is probably affected more by the protein deficiency than is the carcase

Table 11 a. Non-haemin iron and plasma iron in growing rats on a diet containing 22 % protein and with a daily iron intake of 280 or 560 µg.

	Group A I, 560 µg iron per day	Group A II, 280 µg iron per day	D_{AI-AII}	P
Non-haemin iron in liver, mg per 100 g fresh tissue	3.17[1]	2.79[1]	0.38	<0.5
Non-haemin iron in spleen, mg per 100 g fresh tissue	22.61[1]	8.93[1]	13.68	<0.05
Non-haemin iron in carcase, mg per 100 g fresh tissue	1.09[1]	0.94[1]	0.15	<0.2
Plasma iron, µg per 100 ml . . .	231	201	30	<0.4

[1] The female in each group excluded.

protein. The spleens varied considerably in weight from one group to another, especially in the case of the 22 % animals, the cause not being clear. Owing to these variations it is difficult to compare the spleen-weights of the low-protein animals and the 22 % animals.

Data concerning the non-haemin iron in the 22 % animals and the low-protein animals on 280 and 560 µg of iron per day are analysed further in Tables 11 a and 11 b. The last animal in each group, the female, has been excluded, because the non-haemin iron in female rats is much higher than in males (Widdowson and McCance, 1948, 1953). In the 22 % animals no particular difference was found between the non-haemin iron of the 280 and 560 µg groups: in the group on the larger dose of iron there was at most a slight tendency towards a somewhat higher non-haemin iron level, but only in the case of the spleen is

Table 11 b. Non-haemin iron and plasma iron in growing rats on a low-protein diet and with a daily iron intake of 280 or 560 µg.

	Group B I, 560 µg iron per day	Group B II, 280 µg iron per day	D_{BI-BII}	P
Non-haemin iron in liver, mg per 100 g fresh tissue	13.22[1]	3.81[1]	9.41	<0.01
Non-haemin iron in spleen, mg per 100 g fresh tissue	46.63[1]	12.49[1]	34.14	<0.001
Non-haemin iron in carcase, mg per 100 g fresh tissue	0.90[1]	0.86[1]	0.04	
Plasma iron, µg per 100 ml . . .	142	138	4	

[1] The female in each group excluded.

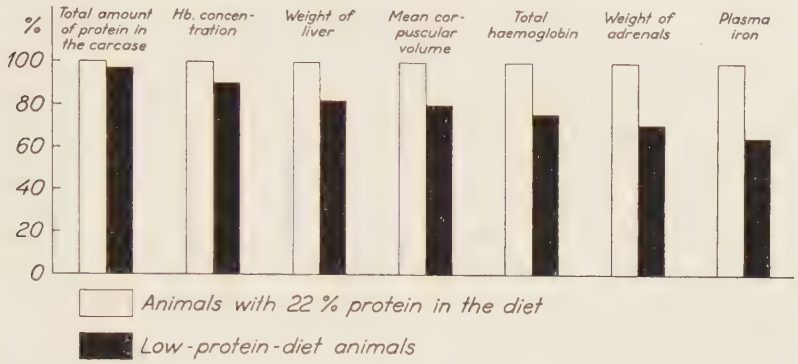

Fig. 7. Comparison between growing rats fed on a diet with 22 % protein and on a low-protein diet.

this difference probably significant. Nor did the plasma iron exhibit any significant difference between the 280 and 560 µg groups, but, as with the non-haemin iron, the mean lay somewhat higher in the group on 560 µg of iron per day.

In the low-protein animals, on the other hand, the iron in the liver and spleen stores was increased on increasing the dose of iron from 280 to 560 µg per day, the 560 µg group having about 3 times as much non-haemin iron in the liver and spleen. The non-haemin iron of the carcase was the same in the two low-protein groups. The increase in non-haemin iron in the liver and spleen in the low-protein group receiving 560 µg was accompanied by no change in the plasma iron, which was the same in both low-protein groups. Comparison of the plasma iron in the 22 % animals with that in the corresponding low-protein groups shows it to be diminished among the latter (216 and 140 µg per 100 ml respectively); this fall in plasma iron due to the protein deficiency is highly significant.

The effect of the protein deficiency on haemoglobin, red blood cells, the weight of the liver and adrenals, and the plasma iron, is represented graphically in Fig. 7.

Summarizing the results of this experiment on the effect of protein deficiency upon growing rats the following can be said. The low-protein animals developed slight, microcytic, normochromic anaemia, the haemoglobin concentration being about 10 % lower than in the animals on adequate protein intake. The total haemoglobin, however, fell by about as much as 25 %, and the quotient g Hb. per g carcase protein by fully 20 %. At the same time the weight of the liver diminished by nearly 20 %, no macroscopic signs of increased deposition of fat being visible. The adrenals diminished in weight on an average by

nearly 30 %, and the plasma iron fell by 35 %. The composition of the carcase was similar in the low-protein animals and those on adequate dietary protein.

In this connection the work of two other teams of investigators deserve mention. Metcoff, Favour, and Stare (1945) found no anaemia in growing rats kept for 10–14 weeks on a diet containing 8 % of casein, but the amount of haemoglobin per unit body surface was significantly diminished. This latter is in agreement with my own finding that the total haemoglobin diminishes more than the carcase protein in growing animals on a low-protein diet. Cartwright and Wintrobe (1948) found an average fall in the serum iron of 32 % during chronic protein deficiency in pigs. The total iron-binding capacity of the serum fell to about $^1/_3$ of the normal.

It is difficult to see why the haemoglobin diminishes in relation to the carcase protein in animals on a protein-deficient diet, but it is tempting to discuss two possibilities. (1) If the turnover of haemoglobin were great in relation to that of the other body proteins in general, this alone would be enough to explain why, during protein deficiency, equilibrium between the building up and breaking down of haemoglobin can only be maintained at a lowered total haemoglobin level. It is, however, possible to increase considerably the amount of haemoglobin synthesized per day by bleeding the animals. The limit for haemoglobin formation set by protein deficiency in non-bled animals is thus not absolute. (2) As the total haemoglobin falls the adrenals diminish in weight. Adrenal insufficiency, e. g. Addison's disease in man or adrenalectomy in animals, carries with it a fall in blood volume; and the blood volume is increased (this increase being occasionally accompanied by polycythaemia) in Cushing's disease, in which there is hyperfunction of the adrenal cortex. It is possible that the diminished total haemoglobin in protein deficiency is secondary to hypofunction of the adrenals, and the low plasma iron, too, could well be explained by adrenal insufficiency. Cartwright *et al.* (1951) have shown that adrenalectomy in dogs is followed by a fall in the plasma iron.

The coefficient of partial correlation between total haemoglobin and adrenal weight after elimination of the variations in carcase protein was calculated for the male rats in the four groups which received 280 or 560 µg of iron per day (low-protein animals and 22 % animals). It was found to be + 0.74, which is highly significant. (The figure for total haemoglobin was lacking in one case, the number of observations thus amounting to 19.) The fact that there is a positive correlation between the total haemoglobin and the weight of the adrenals in the presence of a falling protein intake proves nothing about the cause of the fall in total haemoglobin. We must be content to say that, in the presence of a protein deficiency which produced an almost 30 % fall in the weight of the adrenals, the total haemoglobin diminished more than

the carcase protein, and the plasma iron also fell. Both these phenomena are found in states of adrenal insufficiency.

As previously mentioned, the raising of the iron dose from 280 to 560 µg of iron per day in the low-protein animals resulted in an almost triple increase in the total non-haemin iron of the liver and spleen. Balfour *et al.* (1942), Hahn *et al.* (1943), and Granick (1946) have suggested that the intestinal mucosa is able to accept or reject iron, according to the state of the iron stores in the body. The great increase in absorption of iron which occurs on the above increase in iron intake in animals on a protein-deficient diet suggests that the "mucosa block" (Hahn *et al.*, 1943) is not so effective when the dietary protein is low.

From the work of other investigators it is known that there are exceptions from the "mucosa block" theory. Gubler, Cartwright, and Wintrobe (1949) have shown that absorption of iron is increased during deficiency of pyridoxine. "Since in pyridoxine deficiency the synthesis of haemoglobin is retarded and the serum iron elevated it would be expected that the need of the body for iron would be reduced. Yet iron continues to be absorbed even to the extent that the total body iron is increased." Gubler *et al.* believe that pyridoxine deficiency is an exception from the "mucosa block" theory. Kinney, Hegsted, and Finch (1949) have shown that rats placed on a corn-grit diet with added iron absorb much more iron than do controls. They found that this was primarily due to the low dietary phosphate. Casein added to the corn-grit diet also had a certain limiting effect on absorption of iron. Kinney *et al.* found, too, that it is possible to induce excessive deposition of iron in the livers of animals on a normal diet by adding large amounts of iron salts to the feed. They believe it probable that the normal mechanism of iron absorption is effective only when the iron-phosphorus ratio is within certain limits and the iron intake low. My findings indicate, moreover, that the "mucosa block" is more easily overcome by an increase in iron intake if the diet is at the same time deficient in protein.

Nutritional Iron Deficiency in Growing Rats

The groups on 22 % dietary protein receiving supplementary iron in amounts from 0 to 560 µg per day constitute a pure iron-deficiency experiment in growing animals, in which the development of iron-deficiency anaemia can be followed in relation to progressive lack of iron. The usual haemoglobin-concentration estimations were accompanied by examination of the state of the total haemoglobin, and the changes in the corpuscular constants (mean corpuscular volume, mean corpuscular Hb. concentration, etc.). The plasma iron, and the non-haemin iron of liver, spleen, and carcase, were also determined. By this means it is possible to find out how close the correlation is between

plasma iron and storage iron. The animals on 280 and 560 µg of iron per day have been combined in a single group, since their blood pictures are closely similar. The means for the various groups, the differences between them, and the corresponding *P* values are given in Table 12.

Reduction of the daily iron intake to 70 µg was followed by iron-deficiency anaemia. The haemoglobin concentration fell to 12.5 g per 100 ml, compared with 16.4 g per 100 ml in the animals on 280 and 560 µg of iron per day. (In the following the latter will, for the sake of simplicity, be termed controls.) The total haemoglobin in the 70 µg group amounted to 1.20 g, and in the controls to 1.55 g. The percentage decrease in total haemoglobin and haemoglobin concentration was about the same (22.6 % and 23.8 % respectively). The state of the total haemoglobin in nutritional iron-deficiency anaemia thus differs basically from that in low-protein anaemia, in which the changes in the total haemoglobin are greater than the corresponding changes in haemoglobin concentration. The haematocrit reading in the 70 µg group diminished from 46 % to 39 %, the number of red blood cells remaining unchanged. The mean corpuscular volume fell from 69 to 56 cu. microns, the mean corpuscular haemoglobin from 25 to 18 micromicrograms, and the mean corpuscular Hb. concentration from 36 % to 32 %. The pure nutritional iron-deficiency anaemia is thus of microcytic, hypochromic type.

When the iron deficiency became even severer, as in the groups on 35 and 0 µg of iron per day, the anaemia became progressively more profound. The haemoglobin concentration in the 35 µg group fell to 8.4 g per 100 ml, and the total haemoglobin to 0.81 g — the total haemoglobin was thus still falling to the same extent as the haemoglobin concentration (by 47.7 % and 48.8 % respectively). The haematocrit reading in the 35 µg group fell to 27 %, the red cell count remaining unchanged. The microcytosis thus became more marked, the mean corpuscular volume having fallen to 40 cu. microns. The mean corpuscular haemoglobin fell to 12 micromicrograms, but the mean corpuscular Hb. concentration in the 35 µg group did not diminish beyond the level reached in the 70 µg group.

In the group not given any supplementary iron we find the most severe anaemia of the series, the haemoglobin concentration being only 6.0 g per 100 ml. The fall in total haemoglobin continued closely parallel with the fall in haemoglobin concentration. The total haemoglobin in this group amounted to 0.59 g, thus having diminished by 61.9 %. (The corresponding fall in haemoglobin concentration was 63.4 %.) When the iron-deficiency anaemia had reached this degree of severity the number of red blood cells had diminished by about 1 million. The haematocrit reading continued to fall, reaching 21 % in this group, and the mean corpuscular haemoglobin fell to 10 micromicrograms. The difference in mean corpuscular volume between this group

Table 12. Haemoglobin concentration, corpuscular constants, total haemoglobin, non-haemin iron, and plasma iron in nutritional iron-deficiency anaemia.

	I. Animals on 280 or 560 µg Fe per day	II. Animals on 70 µg Fe per day	D_{I-II}	P	III. Animals on 35 µg Fe per day	D_{II-III}	P	IV. Animals receiving no iron salt	D_{III-IV}	P
Number of animals	12	6			6			6		
Hb. concentration, g per 100 ml	16.4	12.5	3.9	< 0.001	8.4	4.1	< 0.001	6.0	2.4	< 0.01
Red blood cells, millions/mm³	6.7	6.0			6.9			5.8	1.1	< 0.05
Haematocrit reading, %	46	39	7	< 0.01	27	12	< 0.001	21	6	< 0.01
Mean corpuscular volume, cu. microns	69	56	13	< 0.01	40	16	< 0.001	36	4	< 0.3
Mean corpuscular haemoglobin, micromicrograms	25	18	7	< 0.001	12	6	< 0.001	10	2	< 0.1
Mean corpuscular Hb. concentration, %	36	32	4	< 0.02	31	1	< 0.5	29	2	< 0.3
Total haemoglobin, g	1.55	1.20	0.35	< 0.01	0.81	0.39	< 0.001	0.59	0.22	< 0.02
Plasma iron, µg per 100 ml	216	60	156	< 0.001	41	19	< 0.2	28	13	< 0.1
Non-haemin iron in liver, mg per 100 g fresh tissue	2.98[1]	0.91	2.07	< 0.001	0.92			0.93		
Non-haemin iron in spleen, mg per 100 g fresh tissue	15.77[1]	3.14	12.63	< 0.01	2.34	0.80	< 0.2	2.69		
Non-haemin iron in carcase, mg per 100 g fresh tissue	1.02[1]	0.54	0.48	< 0.001	0.45	0.09	< 0.1	0.45		

[1] The female in each group has been excluded.

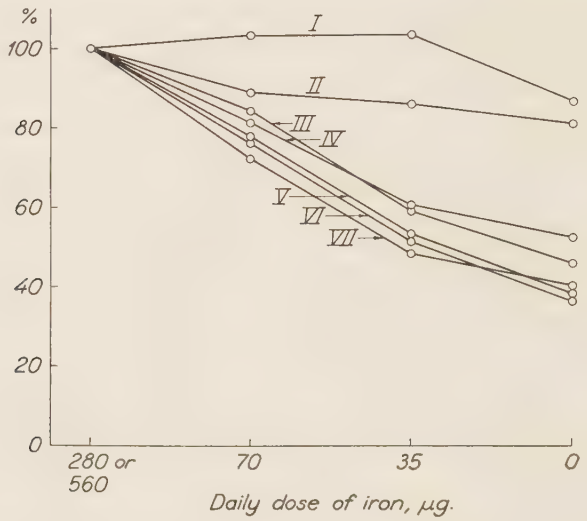

Fig. 8. Changes in haemoglobin concentration, total haemoglobin, and corpuscular constants, in nutritional iron-deficiency anaemia.

I. Red cell count
II. Mean corpuscular Hb. concentration
III. Haematocrit reading
IV. Mean corpuscular volume
V. Total haemoglobin
VI. Haemoglobin concentration
VII. Mean corpuscular haemoglobin.

without supplementary iron and the 35 µg group is, like the difference in mean corpuscular Hb. concentration, not significant. The *P* value for the difference in mean corpuscular haemoglobin is only slightly greater than 0.05.

The changes in haemoglobin concentration, red cell count, total haemoglobin, and the corpuscular constants, during increasing iron deficiency are represented schematically in Fig. 8. The means for the different groups are expressed in per cent of the corresponding means for the group receiving 280 or 560 µg of iron per day.

Fig. 8 shows plainly how closely the changes in haemoglobin concentration and total haemoglobin follow each other during progressive iron deficiency. The mean corpuscular haemoglobin curve also follows these two, but in the group with no supplement it appears to flatten out, as does the mean corpuscular volume curve. In all the iron-deficiency groups the haematocrit reading and mean corpuscular volume diminished rather less than the haemoglobin concentration: in the unsupplemented group the difference between the percentage decrease in haemoglobin

concentration and haematocrit reading is probably significant, and between the percentage fall in haemoglobin concentration and mean corpuscular volume it is significant. The red cell count and mean corpuscular Hb. concentration curves follow a deviating course. The number of red cells diminishes only in the unsupplemented group. The mean corpuscular Hb. concentration fell in the 70 µg group, but the further fall on increasing iron deficiency proceeds very slowly, and cannot be established statistically with the small groups of this experiment. This fall in mean corpuscular Hb. concentration is, however, considered to be specific for iron-deficiency anaemia.

In the groups in which iron-deficiency anaemia developed, the plasma iron was greatly diminished: in the 70 µg group it amounted to 60 µg per 100 ml, compared with the value 216 µg per 100 ml in the controls. There was no longer any storage iron in the liver and spleen of the animals in the 70 µg group: by reducing the dose of iron to 35 or 0 µg per day it was impossible to reduce further the hydrolysable iron in the liver and spleen, the storage iron of these organs already being spent in the 70 µg group (see Table 8 a). The hydrolysable iron of liver and spleen in the three iron-deficiency groups was thus not true storage iron, and this quantity must be subtracted from the hydrolysable iron of the 280 and 560 µg groups to obtain their content of true storage iron.

The carcase contained rather more storage iron in the animals of the 70 µg group than in those on 35 and 0 µg of iron per day. If the animals in the two lowest groups are combined into a single group the difference between the non-haemin iron of the carcase in this group and the 70 µg group is significant. The non-haemin iron of the carcase in the 35 and 0 µg groups must be subtracted from the non-haemin iron of the 70, 280, and 560 µg groups in order to obtain the latter groups' content of true storage iron.

The plasma iron of the 35 and 0 µg groups was even lower than in the 70 µg group. The plasma iron in the 0 µg group was 28 µg per 100 ml, the difference between this and the 70 µg group being probably significant ($P < 0.02$). The lowering of the plasma iron and of storage iron in liver and carcase on progressive iron deficiency is demonstrated in Fig. 9. Concerning the correlation between plasma iron and storage iron it is clear from this diagram that the plasma iron is markedly diminished when the liver no longer contains any storage iron. On pressing the iron deficiency still further — from the point when the liver stores are spent — the fall in plasma iron appears to parallel the diminution in the storage iron of the carcase. (The difference between the plasma iron of the groups on 280 and 560 µg of iron per day is not significant.)

Access to a certain amount of storage iron would thus appear to be an important factor in the maintenance of the plasma iron level. The current view on the renewal of plasma iron is that it is utilized in a

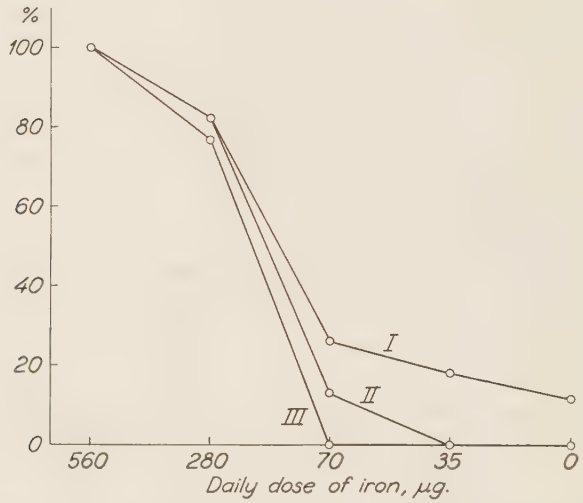

Fig. 9. Plasma iron, and storage iron in liver and carcase, in iron-deficiency anaemia.

I. Plasma iron
II. Storage iron concentration in carcase
III. Storage iron concentration in liver.

close cyclical process (haemoglobin–plasma iron–haemoglobin) to a great extent independent of absorption, excretion, and storage (see Laurell, 1952). Since in the 70 µg group there is also anaemia simultaneously with the fall in plasma iron and the exhaustion of the storage iron in liver and spleen, we must consider the possibility of whether the reduction in the daily turnover of haemoglobin could not alone be responsible for the fall in plasma iron. In the 70 µg group the reduction in total haemoglobin amounts to about 25 %, and 25 % less haemoglobin iron is metabolized per day than in the controls, on condition that the life-span of the red cells is the same. If the iron from the daily turnover of haemoglobin alone was enough to maintain the plasma iron at a normal level, it is hardly to be expected that a 25 % decrease in haemoglobin turnover would reduce the plasma iron to as little as 60 µg per 100 ml, viz. 28 % of the control level. When the iron stores are completely exhausted the plasma iron probably derives directly from the breaking down of haemoglobin and other haemin compounds. The storage iron is already spent in the 35 µg group, with a 50 % decrease in the total haemoglobin. Here the iron from the daily turnover of haemins can only maintain a plasma iron level of about 40 µg per 100 ml. Thus access to a certain amount of storage iron would appear necessary for the maintenance of a normal plasma iron level.

If the organism possesses sufficient storage iron the dietary iron is of no great importance in the regulation of the plasma iron. This can be seen from the experiment on adult male rats in which the plasma iron was normal in spite of their having been kept on a practically iron-free diet during several weeks.

The disappearance of storage iron from the liver and spleen and the simultaneous fall in plasma iron would appear to be early signs of nutritional iron deficiency. This is also in accordance with the clinical observation that cases of sideropaenia without anaemia may occur (Jasiński, 1949, 1950; Roth, 1950; and others).

A rat liver in which the storage iron is exhausted contains about 0.9 mg of hydrolysable iron per 100 g of tissue. The corresponding figure for spleen is 2–3 mg per 100 g of tissue: this higher value may be due to iron liberated from old erythrocytes. Whatever the position in the spleen, the liver always contains a fraction of non-haemin iron which is not storage iron. In this connection the question also arises of the character of that part of the hydrolysable iron in the carcase which is not mobilizable storage iron. As can be seen from the following, it cannot be haemin-bound iron split off during the hydrolysis. No iron could be liberated from pure myoglobin on treatment with 5 N HCl at 95° C during one hour (see Methods). Cytochrome c, too, is very stable on treatment with acid (Theorell and Åkesson, 1941). Catalase and peroxidase are present in very small quantities in the exsanguinated carcases. After viviperfusion only about 2 % of the animal's blood remains in the carcase, and only about 16 μg of iron is liberated per ml of blood during the acid-extraction used in determining the non-haemin iron. During treatment of the homogenized carcase with acetone to remove the water some of the remaining haemoglobin passes into the acetone-water mixture; the hydrolysable iron is then determined on the dry powder obtained by treating the homogenized carcase with acetone and ether. Only a minute amount (a few μg) of the hydrolysable iron in the carcase can thus derive from haemoglobin or other haemins.

The hydrolysable iron of the carcase includes the hydrolysable iron of the bone-marrow. Analyses of isolated bone-marrow from femur showed that the storage iron of the bone-marrow paralleled that of the liver during iron deficiency. When all storage iron was spent the non-haemin iron of the liver amounted to 0.090 mg; and the non-haemin iron in the bone-marrow can be assumed to be of the same order. The whole of the hydrolysable iron in the carcase which could not be utilized for haemoglobin synthesis amounted to *c.* 0.75 mg. The non-haemin iron of the bone-marrow thus constitutes probably no more than 10–15 % of the total amount of hydrolysable iron in the carcase of iron-depleted rats. In conclusion it may be said that the carcase contains some non-haemin tissue iron which is not storage iron. Here it

may be added that, when investigating the storage iron in man, I have found in the brain comparatively large amounts of non-haemin iron which are independent of the storage iron of the body.

Simultaneous Nutritional Iron and Protein Deficiency in Growing Rats

The growing rats kept on a protein-deficient diet supplemented by iron in varying amounts constitute a double-deficiency series in which haemoglobin formation can be studied during simultaneous deficiency of protein and iron. This experiment was carried out firstly to find out whether the amount of storage iron available affects the position of haemoglobin formation in the competition for material for protein synthesis; and, secondly, to establish whether an iron-deficiency anaemia is simply added to the low-protein anaemia in low-protein animals, or whether the two deficiencies have any aggravating or modifying influence upon each other. These points have not been the object of study before.

The different low-protein groups received the same daily dose of iron as the corresponding groups on 22 % dietary iron. The means for the low-protein groups, and the differences between them, with corresponding *P* values, are given in Table 13. The animals on 280 and 560 µg of iron per day have again been collected into a single group, since their blood pictures are closely similar. As previously mentioned, the mean corpuscular Hb. concentration alone shows a significant difference (37 % in the 560 µg group, and 33 % in the 280 µg group), but even in the 280 µg group it seems to be within, or close to, the normal range (cf. 35 % in the 560 µg group of 22 % animals). Since the animals in these low-protein groups were given iron in quantities exceeding the normal requirements of the growing rat, and no important difference in haemoglobin formation existed between them, they have been treated as a single group: this combined low-protein group will in the following be termed "adequate iron" group.

In the 70 µg group the intake of iron was still enough for the formation of the same amount of haemoglobin as in the adequate iron group. On the other hand the haemoglobin concentration fell to 13.5 g per 100 ml compared with 14.7 g per 100 ml in the adequate iron group, a probably significant fall; this was the only fairly certain effect upon the haemoglobin or red cells of the low iron intake in the 70 µg group (see Table 13). There were thus no established differences in total haemoglobin, red cell count, haematocrit reading, or mean corpuscular volume between the 70 µg group and the adequate iron group. The mean corpuscular Hb. concentration was about the same as in the 280 µg group (34 % and 33 % respectively).

Table 13. Haemoglobin concentration, corpuscular constants, total haemoglobin, non-haemin iron, and plasma iron in simultaneous nutritional iron and protein deficiency.

	I. Animals on 280 or 560 µg Fe per day	II. Animals on 70 µg Fe per day	$D_{I\text{-}II}$	P	III. Animals on 35 µg Fe per day	$D_{II\text{-}III}$	P	IV. Animals receiving no iron salt	$D_{III\text{-}IV}$	P
Number of animals	12	6			6			6		
Hb. concentration, g per 100 ml	14.7	13.5	1.2	<0.05	11.2	2.3	<0.02	7.5	3.7	<0.01
Red blood cells, millions/mm³	7.7	7.6			7.6			7.6		
Haematocrit reading, %	42	40	2	<0.2	40			25	15	<0.001
Mean corpuscular volume, cu. microns	55	53	2	<0.4	53			33	20	<0.001
Mean corpuscular haemoglobin, micromicrograms	19	18	1	<0.4	15	3	<0.02	10	5	<0.001
Mean corpuscular Hb. concentration, %	35	34			28	6	<0.05	30		
Total haemoglobin, g	1.18	1.15			0.97	0.18	<0.02	0.60	0.37	<0.001
Plasma iron, µg per 100 ml	140	111	29	<0.1	62	49	<0.001	36	26	<0.01
Non-haemin iron in liver, mg per 100 g fresh tissue		1.10			0.71	0.39	<0.1	0.70		
Non-haemin iron in spleen, mg per 100 g fresh tissue		6.16			3.22	2.94	<0.05	2.68	0.54	<0.3
Non-haemin iron in carcase, mg per 100 g fresh tissue	0.91	0.72	0.19	<0.01	0.49	0.23	<0.001	0.38	0.11	<0.01

On reducing the dose of iron to 35 μg per day the haemoglobin concentration fell to 11.2 g per 100 ml and the total haemoglobin to 0.97 g. The percentage decrease in haemoglobin concentration thus amounts to 23.8 %, and for the total haemoglobin to 17.8 %. As in the 70 μg group, the percentage fall in haemoglobin concentration is greater than the percentage fall in total haemoglobin, but in neither group is the difference significant. The red cell count, haematocrit reading, and thus also the mean corpuscular volume, were still unchanged in the 35 μg group, but the mean corpuscular haemoglobin fell to 15 micromicrograms (cf. 19 micromicrograms in the adequate iron group). The mean corpuscular Hb. concentration also fell significantly from 34 % to 28 %. Thus when iron-deficiency anaemia first developed in the low-protein animals no reduction in the mean corpuscular volume occurred over and above that due to the protein deficiency, but the haemoglobin saturation of the red cells diminished; i.e., the appearance of the iron-deficiency anaemia was associated, as in the case of the 22 % animals, with a fall in the mean corpuscular Hb. concentration, which is considered to be a specific sign of iron-deficiency anaemia.

The animals that received no iron supplements developed, as expected, the severest anaemia of the low-protein groups, with a haemoglobin concentration of 7.5 g per 100 ml (a reduction of 49 %), the total haemoglobin amounting to 0.60 g (a fall of 49.2 %). The percentage decrease in haemoglobin concentration and total haemoglobin are thus equal. The red cell count was still unchanged, the mean corpuscular haemoglobin having fallen to the same extent as the haemoglobin concentration. The haematocrit reading decreased from 42 % to 25 %, a fall amounting to about 40 % of the mean value for the adequate iron group. Onto that fall in mean corpuscular volume due to protein deficiency is thus superimposed a further decrease produced by the iron deficiency. The mean corpuscular Hb. concentration (average 30 %) was slightly higher than in the 35 μg group (average 28 %), but the difference is not significant.

As with the 22 % animals, we find that the mean corpuscular Hb. concentration is definitely reduced when the iron deficiency has produced a fall in haemoglobin concentration by 20–25 %. On pressing the iron deficiency still more, little or no further fall in mean corpuscular Hb. concentration takes place.

Fig. 10 demonstrates the changes in haemoglobin concentration, total haemoglobin, haematocrit reading, mean corpuscular haemoglobin, mean corpuscular volume, and mean corpuscular Hb. concentration, in the low-protein animals on increasing iron deficiency: all values are expressed in per cent of the corresponding mean for the adequate iron group. It can be seen how the haemoglobin concentration, total haemoglobin, and mean corpuscular haemoglobin diminish parallel with each other on increasing iron deficiency within the limits of this experiment. The

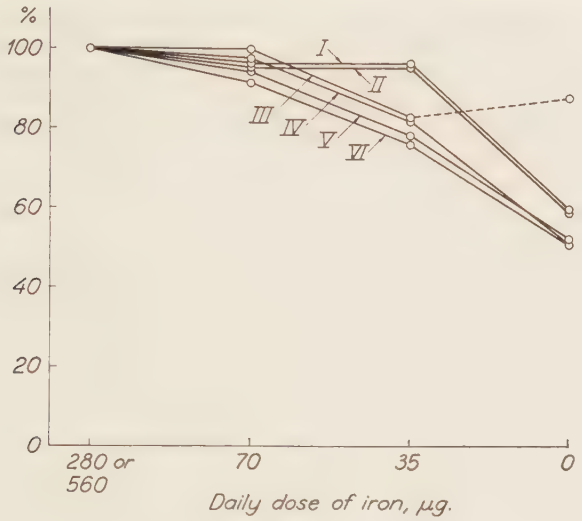

Fig. 10. Changes in haemoglobin concentration, total haemoglobin, and corpuscular constants, in simultaneous nutritional iron and protein deficiency.

I. Mean corpuscular volume
II. Haematocrit reading
III. Mean corpuscular Hb. concentration
IV. Total haemoglobin
V. Mean corpuscular haemoglobin
VI. Haemoglobin concentration.

curves for mean corpuscular volume, haematocrit reading, and mean corpuscular Hb. concentration deviate from the other three. When iron-deficiency anaemia is superimposed upon low-protein anaemia the mean corpuscular Hb. concentration first falls, but this fall is not accompanied by any further diminution in haematocrit reading or mean corpuscular volume beyond that due to the protein deficiency. The curve for the red cell count has been excluded from this diagram owing to lack of space. As mentioned previously the iron-deficiency anaemia in the low-protein animals of this experiment never reached the point where the red cell count falls. This, however, was the case in the 22 % animals in the group without iron supplement.

We shall now consider the storage iron in low-protein animals during iron deficiency (see Tables 9 a and 13). In the 70 µg group the liver and spleen contain small amounts of storage iron. The difference in liver non-haemin iron (mg per 100 g fresh tissue) between this group and the combined 0 and 35 µg groups is probably significant; this is also so in the case of the non-haemin iron of the spleen. In the groups on

0 and 35 μg of iron per day the liver no longer contains any storage iron: the non-haemin iron of the liver has already reached a minimum in the 35 μg group, and is not depressed further by the severer iron deficiency and anaemia in the group without iron supplement. Thus the hydrolysable iron in the 0 and 35 μg groups cannot be storage iron. When the storage iron of the liver is completely exhausted about 0.70 mg of non-haemin iron per 100 g of fresh tissue remains: the spleen, at this point, contains about 3 mg of hydrolysable iron per 100 g of tissue. These quantities must be subtracted from the non-haemin iron in the 70, 280, and 560 μg groups to obtain the corresponding figures for storage iron: for the liver these are 0.40 mg, 3.67 mg, and 12.07 mg, respectively, and for the spleen 3 mg, 14 mg, and 46 mg, respectively (average).

Although the storage iron of the liver and spleen varied from very low levels in the 70 μg group to about 12 mg per 100 g liver tissue and 46 mg per 100 g splenic tissue in the 560 μg group, there was no difference in total haemoglobin between the 70, 280, and 560 μg groups. The amount of storage iron available in liver and spleen would thus appear to be of no significance for the position of haemoglobin formation in the competition for material for protein synthesis. A surplus of storage iron exerts no stimulating effect on haemoglobin formation during protein deficiency. Even when the liver and spleen contain only traces of storage iron, as in the 70 μg group, the same amount of haemoglobin is formed as when there is a surplus of iron in these stores.

The carcase in the 280 and 560 μg groups contained 0.91 mg of non-haemin iron per 100 g of fresh tissue, in the 70 μg group 0.72 mg, in the 35 μg group 0.49 mg, and in the group without supplement 0.38 mg. In the last group the iron deficiency was so severe that the haemoglobin concentration fell to 7.5 g per 100 ml. The storage iron of the carcase was probably exhausted in this group, the carcase containing 0.38 mg of non-haemin iron per 100 g of fresh tissue compared with 0.40 mg in the corresponding group of 22 % animals. This is not storage iron, and should be subtracted from the non-haemin iron of the other groups to obtain their true content of storage iron. The resulting figures are as follows: in the 560 μg group, 0.53 mg of iron per 100 g of carcase; in the 280 μg group, 0.53 mg; in the 70 μg group, 0.34 mg; and in the 35 μg group, 0.11 mg. Whereas the storage iron of liver and spleen in the 70 μg group is almost completely spent, the storage iron of the carcase has only diminished by about $^1/_3$. We thus find, as in the 22 % animals, that the liver and spleen contain stores of iron which are readily mobilized when required. In contrast to the storage iron of liver and spleen, the greater part of the storage iron of the carcase is utilized parallel with, and not before, the development of an iron-deficiency anaemia.

The plasma iron in the low-protein animals on 280 or 560 μg of iron per day (adequate iron group) was on an average 140 μg per

100 ml. As previously mentioned, the plasma iron in these animals is reduced compared with that in the corresponding animals on 22 % dietary protein (216 µg per 100 ml). In the low-protein group on 70 µg of iron per day the plasma iron was on an average 111 µg per 100 ml. The difference between the plasma iron in this and the adequate iron group is not significant. The lower plasma iron in the 70 µg group may be due to chance, but on the other hand the iron deficiency was so great that at least two of the animals lacked true storage iron in the liver. There would, however, appear to be no great difference in plasma iron between low-protein animals with only small amounts of storage iron in liver and spleen and those with a great surplus. Within these limits there would thus appear to be no correlation between the plasma iron and the size of the iron stores. In the 35 µg group the liver and spleen no longer contain any storage iron, the plasma iron having fallen to 62 µg per 100 ml. This fall in plasma iron, in comparison with that in the low-protein groups on higher iron intake, is highly significant. In iron deficiency a marked fall in plasma iron occurs just as the storage iron of liver and spleen disappears. At this degree of iron deficiency the storage iron of the carcase is diminished by about $^1/_3$. The low-protein group without iron supplement has the lowest of the plasma iron levels, mean 36 µg per 100 ml, and the difference between this group and the 35 µg group is significant. An iron deficiency more severe than that corresponding to the stage when the storage iron of liver and spleen is just exhausted seems to carry with it in the low-protein animals, too, a further fall in plasma iron. As was pointed out above, the greater part of the storage iron of the carcase was only utilized when the stores of liver and spleen had been used up.

Fig. 11 shows the changes in the plasma iron and storage iron in liver and carcase in low-protein animals on increasing iron deficiency. The mean for each group is expressed in per cent of the corresponding mean for the 560 µg group. The figures for the 70 µg group might be misleading: at least two of the animals had no storage iron in the liver, and the more advanced iron deficiency in these may have had the effect of lowering the mean for plasma iron in this group. Bearing this in mind, the diagram in Fig. 11 clearly shows that the main fall in plasma iron takes place just as the storage iron of liver and spleen disappears. In the presence of still more severe iron deficiency the fall in plasma iron curve parallels that of the storage iron of the carcase.

A comparison will now be made of the different corpuscular constants in the low-protein animals and the 22 % animals without iron supplement. The haemoglobin concentration in the latter was 6.0 g per 100 ml, and in the low-protein animals 7.5 g per 100 ml: although the haemoglobin concentration was 20 % lower in the 22 % animals, the two unsupplemented groups may be said to have an iron-deficiency anaemia of approximately the same degree of severity. Fig. 8 leaves the im-

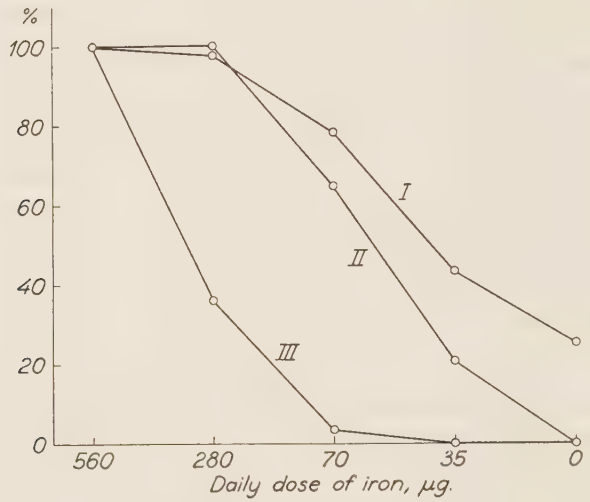

Fig. 11. Plasma iron, and storage iron in liver and carcase, in simultaneous nutritional iron and protein deficiency.

I. Plasma iron
II. Storage iron concentration in carcase
III. Storage iron concentration in liver.

pression that the mean corpuscular haemoglobin and mean corpuscular volume approach asymptotically a limit when the iron deficiency becomes as severe as in the unsupplemented 22 % group. In the 22 % group the mean corpuscular volume and mean corpuscular haemoglobin probably lay in the vicinity of the lower limit for these constants, the mean corpuscular volume being 36 cu. microns (31–45) and the mean corpuscular haemoglobin 10 micromicrograms (9–12). In the low-protein group without iron supplement the mean corpuscular volume was 33 cu. microns (31–35) and the mean corpuscular haemoglobin 10 micromicrograms (9–11). The mean corpuscular Hb. concentration in the 22 % animals was 29 % (25–33) and in the the low-protein group 30 % (27–32). The red blood cells in low-protein animals with iron-deficiency anaemia have thus approximately the same mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular Hb. concentration as in animals on adequate protein intake and with iron-deficiency anaemia of approximately the same degree of severity. Iron-deficiency anaemia in low-protein animals has thus the same characteristics as in animals on adequate protein intake.

Finally, it may be said that nutritional iron-deficiency anaemia develops in the same way in low-protein animals as in animals on an adequate intake of protein; and there is nothing to suggest that it would

be aggravated or modified in any other respect by a simultaneous deficiency of protein. Since the low-protein animals were on experiment longer than the 22 % animals it cannot be said from this experiment whether the protein content of the diet has any influence upon the absorption of iron when there is little dietary iron.

Summing up the results of these experiments concerning the influence upon haemoglobin formation of varying the intake of iron and protein in growing animals, the following may be said.

When the intake of iron is adequate, a low dietary protein results in only a slight fall in the haemoglobin concentration, whereas the total haemoglobin shows a marked fall compared with controls of similar body-weight. The low-protein anaemia is microcytic and normochromic or slightly hypochromic. The total haemoglobin and the adrenal weight are reduced in relation to the carcase protein. After eliminating the variations in carcase protein the coefficient of partial correlation between total haemoglobin and adrenal weight was found to be + 0.74. In protein deficiency the plasma iron is low even when the iron stores are abundant.

In the groups on adequate protein intake but inadequate intake of iron the development of pure nutritional iron-deficiency anaemia can be followed in detail. Here the percentage fall in haemoglobin concentration is as great as that of the total haemoglobin. This applies also to the mean corpuscular haemoglobin, at any rate down to about 40 % of the normal, whereas the mean corpuscular volume falls more slowly. The mean corpuscular Hb. concentration falls sharply at the onset of the anaemia, but hardly decreases further as the anaemia progresses. The plasma iron begins to show a marked fall just as the last remnants of storage iron in liver and spleen are used up. Some of the storage iron in the carcase is still untouched at this stage, and is now drawn upon as the anaemia progresses. Both the parenchymatous organs and the carcase contain a fraction of non-haemin iron which can evidently not be used in haemoglobin synthesis.

When the iron intake was varied in animals on a low-protein diet it became obvious that the supply of storage iron has no influence upon the position of haemoglobin in the competition for protein. The limiting effect of protein deficiency upon haemoglobin formation is not greater when the supply of storage iron is short, nor does a surplus of storage iron stimulate haemoglobin formation in protein deficiency. When the iron intake is low enough to produce an iron-deficiency anaemia, this is simply superimposed upon the low-protein anaemia and develops in the same way as in the animals with adequate protein intake. The appearance of the iron-deficiency anaemia is marked here, as in the animals with adequate protein intake, by a distinct fall in the mean corpuscular Hb. concentration.

CHAPTER V

Haemoglobin Formation in Pregnant and Lactating Rats on Varying Intake of Protein

When new requirements for material for protein synthesis appear in animals on a low protein intake the competition for this material becomes heightened, and it is possible that haemoglobin formation may suffer as a result. Requirements which can be thought to have especially high priority in this competition include pregnancy, lactation, malignant tumours, active antibody formation, etc. In investigating the position of haemoglobin formation during states of heightened competition for raw materials for protein synthesis, my studies have been limited to pregnancy and lactation.

Haemoglobin formation in the presence of protein deficiency during pregnancy and lactation has not previously been the object of systematic investigations. Bethell (1936) described cases of anaemia in pregnant women where the anaemia was not affected by iron therapy but was improved by protein-rich diet. This anaemia, which he believed to be due to protein deficiency, was characterized by a normal colour index and by red cells of normal or increased volume. In the same paper Bethell mentioned that Miss Jean Kyer had found that pregnant rats on a low-protein diet with a surplus of iron developed severe anaemia with red cells of increased volume: no details of this work can be found.

Experimental

Nulliparous female rats about 4 months old and weighing 180–243 g were used. For mating purposes four females and one male were kept in the same cage during one week, and were placed on a synthetic diet containing between 4.5 and 22 % of protein. (The composition of the diet was in other respects the same as that used in the preceding experiments, except that the content of water-soluble vitamins was doubled.) Together they were given 100 g of feed and 5 mg of iron per day. A group on 9 % protein and another on 22 % protein, together with two of the animals from the 4.5 % group, received no iron during the whole period of the experiment. The females were placed in indi-

Table 14. Daily intake of protein and iron during pregnancy and lactation in the various groups.

Group No.	Size of group	Daily intake of feed, g	Protein in the diet, %	Daily intake of protein, g	Daily intake of iron, mg
I	8	28	22	6.16	1
II	7	22	22	4.84	0
III	7	28	14	3.92	1
IV	4	32	12	3.84	1
V	7	18	9	1.62	0
VI	8	22	6	1.43	1
VII	8	22	4.5	0.99	1

Two of the animals in Group VII were given no iron during the experiment.

vidual cages after mating. As soon as gestation could be suspected on the grounds of weight-gain the ration was increased to 22–28 g. The animals are grouped according to the amount of feed they received, its content of protein, and the daily dose of iron (see Table 14): the groups are arranged numerically after the falling daily consumption of protein.

Groups II, V, VI, and VII received a smaller quantity of feed than Groups I, III, and IV. This is because the former experiments were performed first, and because the animals in the two lowest protein groups (V and VII, with 4.5 and 6 % dietary protein) seemed to be on an *ad lib.* consumption of feed when offered about 22 g per day. On increasing the protein content of the diet it became clear that this ration was too small. The quantity of feed per day in Groups I and III was therefore increased to an amount which better corresponded to the animals *ad lib.* consumption. In these two groups exact pair-feeding was carried out. Group IV was given the largest quantity of feed in order to see whether it was possible to save on protein by supplying a certain surplus of calories.

The experiments were broken off when the young were 21–25 days old. The following determinations were done on each dam: haemoglobin concentration; total haemoglobin; weight of adrenals; carcase protein; and non-haemin iron in liver, spleen, and carcase. The plasma iron, too, was estimated in Groups I, III, and VII. The results are given in Table 15 a. (In addition the haematocrit reading and red cell count were done in Groups I, VI, and VII.)

The haemoglobin concentration was determined in each of the young; and estimations of the total amount of protein and the non-haemin iron of the livers and the carcases were done for each litter. The plasma iron was determined as often as it was possible to get enough plasma, the blood being collected from the jugular veins of all the animals of

Table 15 a. Haemoglobin concentration, total haemoglobin, carcase protein, weight of adrenals, plasma iron, and non-haemin iron in rats after pregnancy and lactation, the animals having been kept on a protein-deficient diet.

Rat No.	Body-weight at mating, g	Decrease or increase in weight during pregnancy and lactation, g	Hb. concentration, g per 100 ml	Total haemoglobin, g	Plasma iron, µg per 100 ml	Non haemin iron in liver, mg	Non haemin iron in spleen, mg	Non haemin iron in carcase, mg	Protein in the carcase, g	g Hb. per g protein in the carcase	Weight of adrenals, mg
						Figures in brackets indicate mg non-haemin iron per 100 g fresh tissue					
Group I. 22% protein in the diet. Daily intake 28 g food and 1 mg iron.											
1	225	− 6	15.5	1.90	302	1.089 (7.26)	0.291 (42.2)	2.624 (1.74)	24.8	0.077	45.2
2	218	+ 3	14.1	1.61	246	1.969 (12.83)	0.505 (77.7)	2.687 (1.72)	27.3	0.059	52.8
3	208	+29	17.1	1.92	388	0.989 (5.19)	0.187 (26.3)	2.379 (1.38)	25.9	0.074	40.9
4	208	+15	18.5	2.30	271	0.780 (4.52)	0.453 (63.8)	2.605 (1.57)	26.1	0.088	51.5
5	242	−16	14.1	1.93		0.647 (3.30)	0.237 (30.0)	2.449 (1.54)	26.9	0.072	59.3
6	231	− 16	15.0	1.66	334	0.647 (5.78)	0.278 (33.5)	1.655 (1.00)	26.5	0.063	50.4
7	231	− 1	15.9	1.73	212	1.199 (6.88)	0.426 (40.6)	2.454 (1.58)	25.1	0.069	53.8
8	236	+41	15.8	2.25	284	1.650 (13.15)	0.435 (41.8)	3.154 (1.55)	31.4	0.072	57.4
Mean	225	+ 6	15.8	1.91	291	1.121 (7.30)	0.352 (44.5)	2.501 (1.51)	26.8	0.072	51.4
Group II. 22% protein in the diet. Daily intake 22 g food and no iron.											
9	200	−10	16.8	1.43		0.272 (2.18)	0.076 (22.4)	1.754 (1.33)	20.7	0.069	47
10	235	−20	14.4	1.59		0.461 (3.86)	0.787 (112.4)	2.234 (1.35)	27.8	0.057	52
11	228	−34	14.4	1.57		0.435 (3.75)	0.489 (76.4)	2.931 (2.05)	27.2	0.058	48
12	222	− 8	14.4	1.74		0.233 (2.10)	0.293 (43.7)	2.057 (1.29)	31.1	0.056	46
13	243	−43	12.2	1.57		0.390 (3.17)	0.366 (65.4)	3.315 (2.27)	24.9	0.063	46
14	221	−21	13.0	1.38		0.341 (2.64)	0.180 (40.0)	2.923 (1.99)	22.5	0.061	36
15	236	−51	11.6	1.39		0.597 (5.17)	0.412 (82.6)	2.206 (1.71)	23.3	0.059	54
Mean	226	−27	13.8	1.52		0.390 (3.27)	0.372 (63.3)	2.489 (1.71)	25.4	0.060	47
Group III. 14% protein in the diet. Daily intake 28 g food and 1 mg iron.											
16	231	−49	14.2	1.40	259				21.7	0.065	33.2
17	214	−58	14.2	1.20	143				20.4	0.059	35.7
18	198	−39	10.7	1.08	116				21.5	0.050	32.2
19	222	−35	14.4	1.29	145				22.3	0.058	37.3
20	199	− 4	14.1	1.42	104				25.7	0.055	44.2
21	239	−54	12.4	1.21	75				24.4	0.050	35.7
22	192	+ 1	14.8	1.35	155				25.7	0.053	37.1
Mean	214	−34	13.5	1.28	142				23.1	0.056	36.5

Group IV. 12% protein in the diet. Daily intake 32 g food and 1 mg iron.											
23	227	+ 2	10.6	1.30					26.8	0.048	51.2
24	236	−58	14.2	1.37					24.2	0.057	38.8
25	224	−20	14.2	1.43					23.1	0.062	38.8
26	225	−31	15.1	1.56					25.5	0.061	35.8
Mean	228	−27	13.5	1.42					24.9	0.057	41.2
Group V. 9% protein in the diet. Daily intake 18 g food and no iron.											
27	197	−34	11.2	0.93		0.734 (5.17)	0.275 (110.0)	2.371 (2.14)	20.6	0.045	34
28	202	−31	11.2	1.08		1.025 (6.10)	0.189 (48.5)	1.776 (1.57)	20.2	0.053	40
29	228	−43	11.2	1.14		1.028 (6.67)	0.578 (120.4)		22.3	0.051	36
30	230	−54	12.4	0.82		1.536 (12.15)	0.598 (139.1)	2.946 (2.27)	21.3	0.039	44
31	221	−42	11.3	1.18		0.955 (6.71)	0.211 (57.0)	2.236 (1.71)	23.3	0.051	38
32	215	−39	12.4	1.08		1.056 (10.54)	0.317 (90.6)	2.525 (2.00)	20.7	0.052	23
33	228	−64	12.5	1.09		0.750 (7.77)	0.366 (74.7)	2.406 (1.81)	23.1	0.047	32
Mean	219	−44	11.8	1.05		1.012 (7.87)	0.362 (91.5)	2.377 (1.92)	21.6	0.048	35
Group VI. 6% protein in the diet. Daily intake 22 g food and 1 mg iron.											
34	214	−47	12.7	1.19		1.271 (9.75)	0.851 (283.7)	2.620 (2.18)	20.5	0.058	33.6
35	218	−51	9.8	0.88		1.573 (10.31)	1.268 (230.6)	2.886 (2.37)	17.7	0.050	36.2
36	222	−67	12.9	0.94		2.875 (33.51)	0.625 (189.4)	2.597 (2.30)	20.6	0.046	33.4
37	223	−65	13.8	1.01		0.840 (7.24)	0.520 (133.3)	3.500 (2.94)	18.9	0.053	34.4
38	218	−55	11.6	0.98		2.817 (22.66)	0.243 (69.4)	2.785 (2.56)	17.9	0.055	26.5
39	222	−65	12.4	0.93		0.381 (3.58)	0.160 (57.1)	2.716 (2.56)	17.3	0.054	31.6
40	231	−84	11.5	0.87		1.648 (16.37)	0.878 (225.1)	2.933 (2.69)	18.5	0.047	31.2
41	242	−77	12.2	0.87		1.428 (12.42)	0.969 (230.7)	3.307 (2.58)	20.6	0.042	32.0
Mean	224	−64	12.1	0.96		1.604 (14.48)	0.689 (177.4)	2.918 (2.52)	19.0	0.051	32.4
Group VII. 4.5% protein in the diet. Daily intake 22 g food and 1 mg iron.											
42	203	−41	10.3	0.82	203	1.467 (10.67)	0.266 (83.1)	2.183 (1.98)	18.5	0.044	28.0
43	230	−57	11.3	0.82	239	0.946 (6.06)	0.322 (100.6)	3.144 (2.54)	20.7	0.040	30.2
44	220	−62	11.0	0.81	136	1.316 (10.05)	0.323 (86.8)	2.547 (2.20)	17.3	0.047	30.5
45	180	−29	10.6	0.92	176	1.044 (8.48)	0.430 (159.3)	2.391 (2.26)	19.6	0.047	30.4
46	198	−53	10.4	0.66		2.333 (25.92)	0.957 (598.1)	3.346 (3.13)	18.7	0.035	27.8
47	202	−51	10.4	0.90	189	1.381 (12.78)	0.748 (249.3)	2.857 (2.53)	19.4	0.046	29.4
48	230	− 8	12.9	1.22		0.706 (5.44)	0.755 (104.9)	2.500 (1.55)	23.5	0.052	40.2
49	226	−54	10.9	0.81	107	0.803 (5.39)	0.498 (121.5)	2.035 (1.70)	20.0	0.041	32.4
Mean	208	−50	10.7	0.82	175	1.327 (11.34)	0.506 (199.8)	2.643 (2.33)	19.2	0.043	29.8

Females Nos. 42 and 44 received no iron supplement. Female No. 48 is not included in the means of the group since she obtained extra protein by devouring her young, the total haemoglobin, protein in the carcase, and adrenal weight thus being higher than those of the others in the group.

Table 15 b. Mean body-weight, mean haemoglobin concentration, total amount of body-protein, mean plasma iron, and mean non-haemin iron in liver and carcase, in the litters of the above dams on weaning.

Litter No.	Size of litter	Mean body-weight, g	Hb. concentration, g per 100 ml	Plasma iron, µg per 100 ml	Non-haemin iron			Total amount of protein in the litter, g
					in liver, mg per 100 g fresh tissue	in carcase, mg per 100 g fresh tissue	total amount in the litter, mg	
Group I. 22 % protein in the diet. Daily intake 28 g food and 1 mg iron.								
1	9	27	9.0	175	1.30	1.03	2.099	33.6
2	10	22	10.4	134	2.60	1.35	2.427	32.9
3	9	33	8.7	126	1.60	0.98	2.284	44.2
4	8	37	10.1		4.00	1.17	2.714	44.8
5	11	23	8.4	74	2.16	1.30	2.806	34.3
6	3	45	6.1	80	1.00	0.85	0.992	18.2
7	8	35	9.8	146	2.45	1.39	3.367	40.0
8	1	70	6.6	198	2.00			
Mean	7.4	37	8.7	133	2.14	1.15	2.384	35.4
Group II. 22 % protein in the diet. Daily intake 22 g food and no iron.								
9	11	15	7.3		0.82	0.74	1.206	25.6
10	3	43	5.7		0.70	0.70	0.889	19.0
11	5	30	6.9		1.48	0.88	1.250	24.1
12	3	47	6.0		0.73	0.70	0.874	19.9
13	6	40	6.9		1.42	0.97	2.157	33.6
14	7	34	4.9		0.80	0.57	1.224	33.8
15	11	23	4.7		1.18	0.65	1.506	36.7
Mean	8	33	6.1		1.02	0.74	1.301	27.5
Group III. 14 % protein in the diet. Daily intake 28 g food and 1 mg iron.								
16	7	26	10.1	61				22.4
17	10	16	11.8	97				24.6
18	7	27	7.7	60				26.6
19	5	29	8.6	122				20.5
20	3	49	9.3	100				19.1
21	4	56	8.6	119				25.4
22	3	39	9.9	76				15.0
Mean	5.6	35	9.4	91				21.9
Group IV. 12 % protein in the diet. Daily intake 32 g food and 1 mg iron.								
23	11							
24	10	25						
25	9	23						
26	7	21						
Mean	9.3	23						

The young in litter No. 23 were killed by the dam.

Litter No.	Size of litter	Mean body-weight, g	Hb. concentration, g per 100 ml	Plasma iron, µg per 100 ml	Non-haemin iron			Total amount of protein in the litter, g
					in liver, mg per 100 g fresh tissue	in carcase, mg per 100 g fresh tissue	total amount in the litter, mg	
Group V. 9% protein in the diet. Daily intake 18 g food and no iron.								
27	10	14	7.3		1.13	1.13	1.555	18.4
28	11	15	6.9		0.61	0.79	1.164	22.6
29	10	15	7.7					21.3
30	8	21	7.7		1.65	0.94	1.316	23.0
31	11	15	7.7		1.54	0.95	1.446	23.6
32	6	23	4.9		0.78	0.67	0.824	18.6
33	5	29	5.0		1.27	0.74	0.961	21.9
Mean	8.7	19	6.7		1.16	0.87	1.211	21.3
Group VI. 6% protein in the diet. Daily intake 22 g food and 1 mg iron.								
34	5	20	9.2	119	3.84	1.22	1.017	13.2
35	7	15	9.9	222	2.17	1.26	0.998	13.4
36	7	13	10.9		2.40	1.18	0.909	12.3
37	6	18	9.3		2.34	1.10	0.907	12.8
38	(8)							
39	11	11	9.8	93	2.68	1.51	1.547	14.8
40	8	14	9.0	210	11.30	1.83	1.992	13.9
41	8	16	8.3	148	5.10	1.37	1.619	15.3
Mean	7.4	15	9.5	158	4.26	1.35	1.284	13.7
The young in litter No. 38 were found dead.								
Group VII. 4.5% protein in the diet. Daily intake 22 g food and 1 mg iron.								
42	9	13	8.1	78	0.94	0.77	0.789	15.1
43	7	14	10.7	143	4.65	1.27	1.163	12.4
44	5	22	5.8	67	1.50	0.77	0.799	15.8
45	3	21	10.1	118	1.50	1.05	0.625	8.7
46	5	14	12.9	148	11.00	1.70	0.952	10.4
47	7	13	12.9	170	10.70	1.85	1.332	13.0
48	(7)							
49	7	11	10.4	144	11.70	1.38	0.805	9.9
Mean	6.1	15						12.2
The dams of litters Nos. 42 and 44 received no iron supplement.								
The young in litter No. 48 were found partly devoured by the dam.								

the litter. The carcase here means the entire body including the skin, after removal of the digestive tract and liver. All the carcases of a litter were homogenized together in a Turmix, and treated as previously described. The protein and non-haemin iron content were estimated on an aliquot of the dry powder obtained from the carcases after treatment with acetone and ether. The non-haemin iron of the liver was determined from a homogenate of all livers in the litter. The results can be seen in Table 15 b. (In the litters from Groups I, VI, and VII the haematocrit reading and red cell count were also determined.)

Results and Discussion

The females in Group I, on 22 % dietary protein and 28 g of feed per day, went through pregnancy and lactation without any great loss in weight, half of the animals being, in fact, heavier at the end of lactation than at mating. The total amount of protein in the carcase was, on an average, 26.8 g (24.8–31.4 g). The total amount of protein in the litters (minus digestive tracts and livers) varied from 18.2 g in litter no. 6 (containing three young) to 44.8 g in litter no. 4 (8 young). The protein in the only rat in litter no. 8 was not determined, but constitutes the minimum for the litters. In most cases more protein was synthesized in the litters during gestation and lactation than was contained in the body of the dam. Since gestation and lactation together occupy a period of only six weeks it is plain that in the rat the growth of the fetus and the subsequent lactation set a tremendous strain on the supply of material for protein synthesis.

If the animals of Group I are compared with the 14 % animals of Group III (the two groups isocalorically fed) the effect on haemoglobin formation during gestation and lactation of moderate protein deficiency can be studied (see Tables 15 a, 15 b, and 16). In contrast to the females of Group I, all females in Group III but one diminished in weight, the mean weight loss for the whole group being 34 g. The carcase protein of the females in Group III amounted on an average to 23.1 g of protein, i.e. about 14 % less than in the females of Group I. The haemoglobin concentration in Group I was 15.8 g per 100 ml, and the total haemoglobin 1.91 g (means). In Group III the haemoglobin concentration had fallen to 13.5 g per 100 ml, and the total haemoglobin to 1.28 g. Whereas the haemoglobin concentration in Group III fell by 14.6 %, the total haemoglobin diminished by 33.0 % of the corresponding mean for Group I: the difference between the percentage decrease in haemoglobin concentration and the percentage decrease in total haemoglobin is highly significant. The quotient g Hb. per g carcase protein in the 14 % animals fell to 0.056 compared with 0.072 in the

Table 16. Comparison on weaning of rats kept during pregnancy and lactation on a daily intake of 6.16 g (Group I) or 3.92 g of protein (Group III).

	Group I	Group III	*D*	*P*
Hb. concentration, g per 100 ml .	15.8	13.5	2.3	<0.02
Total haemoglobin, g	1.91	1.28	0.63	<0.001
Protein in the carcase, g	26.8	23.1	3.7	<0.01
g Hb. per g protein in the carcase	0.072	0.056	0.016	<0.001
Total amount of protein in the litters, g	35.4	21.9	13.5	<0.01
Weight of adrenals, mg	51.4	36.5	14.9	<0.001
Plasma iron, µg per 100 ml . . .	291	142	149	<0.001

22 % animals. The protein deficiency during gestation and lactation thus affected the total haemoglobin more than the other body proteins in general.

The total amount of protein in the litters of the 14 % animals varied between 15.0 and 26.6 g (mean 21.9 g). In spite of the protein-deficient diet it was still possible for the dams to supply material for the synthesis of approximately the same amount of protein in their young as their own carcase contained. This implies a high priority for fetal growth and lactation, but on the other hand it is not so high that protein synthesis in the litters is not limited if the protein content of the dams' diet is reduced to 14 %.

As in my experiments on adult male rats and growing rats it was found in the females of this experiment that, when the diet was deficient in protein, the weight of the adrenals fell as the total haemoglobin diminished. In the 22 % females the adrenals weighed 51.4 mg, and in the 14 % females 36.5 mg (means), i.e. about 29 % less in the latter.

In the growing rats on a protein-deficient diet with a surplus of iron the plasma iron was diminished (see Chapter IV b), and here too we find a fall in plasma iron at the end of lactation in the females that received too little dietary protein. In Group I the plasma iron was on an average 291 µg per 100 ml, and in Group III 142 µg per 100 ml.

The animals in Group IV were on a diet containing 12 % of protein, but received 4 g more feed per day than Groups I and III: the daily consumptions of protein in Groups III and IV were thus very similar. Group IV consists of only four females. There are no great differences between this group and Group III in haemoglobin concentration, total haemoglobin, or the quotient g Hb. per g carcase protein. A dietary protein of 12–14 % is thus definitely too low during gestation and lactation in the rat. Even when the animals feed liberally (as much as they can be got to eat) on such a diet signs of protein deficiency appear.

In the groups with still more severe protein deficiency (9, 6, or 4.5 % dietary protein) the haemoglobin concentration and total haemoglobin continued to fall as the consumption of protein became less. The difference in protein intake between the 9 % animals of Group V and the 6 % animals of Group VI is not so great as might appear from the percentage of protein in the diet, because the former were given only 18 g of feed per day and the latter 22 g per day (see Table 14). The haemoglobin concentration was on an average in the 9 % animals 11.8, in the 6 % animals 12.1, and in the 4.5 % animals 10.7 g per 100 ml; the corresponding total haemoglobin values were 1.05, 0.96, and 0.82 g; and the total amount of carcase protein was 21.6, 19.0 and 19.2 g. The diminution in the carcase protein when the protein content of the diet is reduced to 6 % is already maximum, and is not influenced by further reducing the dietary protein: the females have then lost about 30 % of their carcase protein. In contrast to the carcase protein, the total haemoglobin fell more in the 4.5 % group than in the 6 % group, the difference being probably significant. The quotient g Hb. per g carcase protein was in the 6 % group 0.051 and in the 4.5 % group 0.043.

In the 9 % group, litters nos. 30 and 31 still contained as much as 23.0 and 23.6 g of protein respectively. Although the difference in daily protein consumption between the 9 % animals (1.62 g) and the 6 % animals (1.43 g) is small, the greatest amount of protein synthesized in the litters is about as much as 8 g less in the 6 % group. Litter no. 41 consisting of 8 young contained the greatest amount of protein in the 6 % group, viz. 15.3 g. On reducing the protein intake to a level somewhere between 1.62 and 1.43 g per day (spray-dried hen's egg-white) protein synthesis in the litters falls sharply. (Litters 42 and 44 contained the largest amount of protein in the 4.5 % group—15.1 and 15.8 g respectively.)

The plasma iron values were lower in the dams of the 4.5 % group than in those of Group I, but not lower than in the dams receiving 14 % of protein in the diet.

In association with the heavy protein deficiency the weight of the adrenals fell in the 9 % group to 35 mg, in the 6 % group to 32.4 mg, and in the 4.5 % group to 29.8 mg. The coefficient of partial correlation between total haemoglobin and adrenal weight after elimination of the variations in carcase protein was calculated for all 49 females in this experiment. It was found to be + 0.44, and is significant. Thus, as in the growing rats, a positive correlation is found between total haemoglobin and adrenal weight on varying the protein intake.

The females in Group II that obtained 22 g per day of a diet containing 22 % of protein, and the females in Group V on 9 % of dietary protein, were given no iron during pregnancy and lactation over and above that present in the feed. In spite of this, the liver, spleen, and carcase contained storage iron in such quantities that it would appear

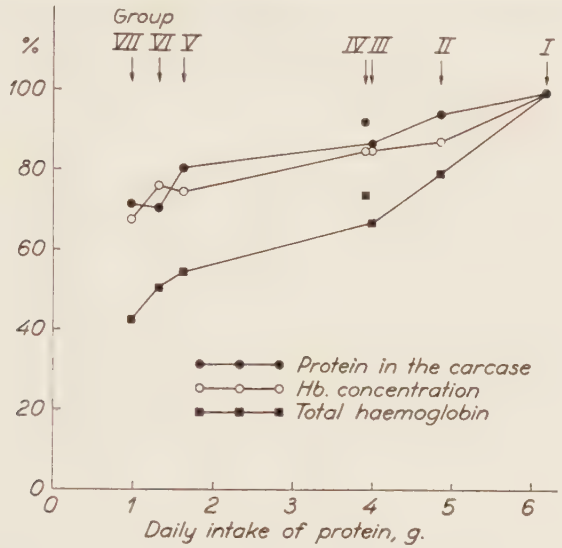

Fig. 12. Changes in haemoglobin concentration, total haemoglobin, and protein in the carcase, during pregnancy and lactation in rats kept on a protein-deficient diet.

unlikely that the supply of iron had limited haemoglobin synthesis in the dams. The animals in Group II obtained per day an amount of protein lying between the consumptions of Groups I and III. The total haemoglobin, the total amount of protein in the carcase, the quotient g Hb. per g carcase protein, and the adrenal weights in Group II also lay between the corresponding values for Groups I and III. The 9 % animals consumed only slightly more protein than those on 6 % dietary protein. The mean values for total haemoglobin and the quotient g Hb. per g carcase protein in the 9 % animals were also fairly similar to the corresponding figures for the 6 % group. The adrenals in the 9 % group weighed on an average 35 mg, and in the 6 % group 32.4 mg. Even in the groups that were not given any supplement of iron it would thus appear that it is the supply of protein alone which has limited haemoglobin formation.

The relationship between the daily protein consumption during pregnancy and lactation in the rat, and haemoglobin concentration, total haemoglobin, and the total amount of protein in the carcase, is shown in Fig. 12: the means for the different groups are expressed in per cent of the corresponding mean for Group I. The animals in the lowest protein groups developed only a moderate anaemia, whereas the total

haemoglobin was greatly reduced: the haemoglobin concentration in the 4.5 % group amounted to 67.7 % of that in Group I, the total haemoglobin being only 42.4 % of the corresponding Group I mean. As can be seen from Fig. 12, the percentage diminution in carcase protein was approximately the same as the percentage reduction in haemoglobin concentration.

The mean life-span of the red corpuscles in female rats has been shown to be 64 days (Berlin and Lotz, 1951). If no haemoglobin were formed during pregnancy and lactation (= 42 days) the total haemoglobin would fall to about 34 % of the initial value, on condition that the life-span of the red cells is the same during pregnancy and lactation in low-protein animals as in normal, non-pregnant female rats. The fall in total haemoglobin in the 4.5 % group is thus almost as great as it would be if no haemoglobin were formed during the experiment. Pregnancy and lactation are thus able to extort material for protein synthesis before the protein requirements for other purposes, to the extent that hardly any protein appears to be left over for the synthesis of haemoglobin in the mother-organism.

In Group I and the low-protein groups on 6 and 4.5 % dietary protein the red cell count and haematocrit reading were also determined, and were found to fall parallel with the fall in haemoglobin concentration in the low-protein groups. There were no differences in mean corpuscular volume, mean corpuscular haemoglobin, or mean corpuscular Hb. concentration between the low-protein groups and Group I. This may appear surprising, since the mild anaemia in the growing animals on a low-protein diet was microcytic. This difference is probably due to the fact that very little haemoglobin and few red cells were formed during pregnancy and lactation in the animals on 6 and 4.5 % dietary protein. Two of the animals in the 6 % group (rats nos. 34 and 37) had a low mean corpuscular volume, and one of these had a higher total haemoglobin value than the others of the group. Unfortunately the haematocrit reading and red cell count were not determined in Group III on 14 % dietary protein.

The percentage content of protein in the carcase of the females did not fall when the animals diminished in weight as a result of protein deficiency in the diet: in Group I it was on an average 16.2 % (14.9–17.5 %), and in Group VII 16.6 % (14.9–18.4 %). It would thus seem impossible for large quantities of protein to be lost without at the same time the other constituents of the tissues being reduced: the composition of the body is thus kept relatively constant. The percentage content of protein in the carcases of the young, on the other hand, was lower in the low-protein groups than in Group I. The mean for Group I was 18.1 %, and for Group VI (with the lowest mean) 15.8 %, the difference being highly significant.

In two of the groups (II and V) the fat-content of the livers of the

dams was also determined: the value for Group II was found to vary between 2.6 and 11.2 % (mean 6.3 %) and that for Group V to vary between 6.0 and 31.2 % (mean 15.5 %). In spite of the great variations within the groups, the difference between them is probably significant. The lower protein intake in Group V compared with Group II thus also resulted in increased deposition of fat in the liver. Even in Group II the daily consumption of protein was, however, too low.

The amount of storage iron in the dams in the various groups has been influenced by several factors. During protein deficiency iron is transferred to the stores from the falling total haemoglobin. In all the animals there is a loss of iron via the placenta to the fetuses, and via the milk to the young. The quantity of this iron varies according to the size of the litter and the weight of the young on weaning. In addition, the animals of Groups II and V, and two from Group VII, obtained no iron in the diet, whereas the rest were given 1 mg per day.

Comparison of the storage iron values for Group I, where the animals received 1 mg of iron per day, with those in Group II, in which the animals obtained no iron, reveals that the content of storage iron in the liver was considerably lower in Group II, the difference in total amount of liver-non-haemin iron between these two groups being as much as 0.731 mg. On the other hand the amount of non-haemin iron in the spleen and carcase was approximately the same in the two groups. In the animals of Group V, which were given no iron, the liver contained almost as much storage iron as in Group I, and considerably more than in Group II; the amounts of storage iron in spleen and carcase were approximately the same as in Groups I and II. That the amount of liver storage iron was larger in Group V than in Group II was mostly due to the fact that the total haemoglobin diminished more in Group V. (The loss of iron to the young was also probably slightly less in Group V: the total amounts of non-haemin iron and the haemoglobin concentrations were approximately the same in the litters of Groups II and V, but the last three litters in Group II were so much heavier than the litters of Group V that they must have contained more haemoglobin and thus more iron.) Whereas the storage iron of the liver varied in Groups I, II, and V, the storage iron of spleen and carcase was very similar in these three groups. This suggests that the iron transferred from mother-organism to fetuses and young is derived in the first place from the stores of the liver.

The total non-haemin iron in the females of Group VI on 6 % dietary protein was greater than in Group I (5.18 mg and 4.00 mg respectively). This is because iron was transferred to the stores from the haemoglobin in the low-protein group, and because the loss of iron to the litters was here less. The sum of the non-haemin iron and haemoglobin iron in the dams plus the non-haemin iron of the litters was, however, greater in Group I than in Group VI (means 12.6 mg and 9.6 mg respectively).

Since the mean weight of the litters was, in addition, more than twice as great in Group I as in Group VI, and the haemoglobin concentration only 10–15 % lower, the young of Group I contained more haemoglobin iron than those of Group VI. The females in Group I thus absorbed considerably more iron during pregnancy and lactation than those in Group VI.

As just mentioned, the sum of the non-haemin iron and haemoglobin iron of the dams and the non-haemin iron of the litters was 9.6 mg (8.0–10.4) in Group VI: the corresponding sum in Group II without supplement of iron was 9.7 mg (8.2–11.6). The haemoglobin concentration of the young was on an average 6.1 g per 100 ml in Group II, and 9.5 g per 100 ml in Group VI. The mean weight of the litters of Group II was 187 g, and the mean protein content 27.5 g; the corresponding figures for Group VI were 106 g and 13.7 g. The haemoglobin concentration was thus 56 % higher in the litters of Group VI than in those of Group II, but at the same time the total amount of body protein was twice as much in the litters of Group II as in those of Group VI: the litters of Group VI certainly contained less haemoglobin iron than the litters of Group II. The females in Group VI thus absorbed little or no iron during pregnancy and lactation, although as much as 1 mg of iron was administered per day. The ample supply of iron in the stores, and the uninterrupted deposition there of iron from the haemoglobin, would appear to be the two factors which in one way or another had a limiting effect on absorption.

Summarizing the results of this experiment concerning the effect of protein deficiency on haemoglobin formation in pregnant and lactating rats the following may be said.

As a result of the increased demands of pregnancy and lactation on material for protein synthesis the position of haemoglobin formation in low-protein animals is further impaired—in a severe protein deficiency to the extent that hardly any haemoglobin is formed during this period. Pregnant and lactating rats on a low-protein diet may lose about 30 % of their carcase protein, but the maximum reduction in the total haemoglobin is much greater, amounting to as much as 55–60 %. The great fall in total haemoglobin here too is accompanied by only a moderate decrease in haemoglobin concentration.

The adrenals diminish in weight in pregnant and lactating low-protein animals; and there is a positive correlation between total haemoglobin and adrenal weight when the protein intake is varied. The plasma iron level falls even when there is a surplus of iron in the stores and the animals are on a high iron intake.

The low dietary protein begins to affect haemoglobin formation in the dams at that state of protein deficiency when the supply of protein also begins to limit the amount of protein synthesized in the litters, and thus also the weight of the litters.

Haemoglobin Formation and Storage Iron in the Litters

The haemoglobin concentration in the weanlings was low in all groups, the means varying from 6.1 g per 100 ml in Group II to 11.4 g per 100 ml for the five litters in Group VII whose dams received iron during pregnancy and lactation. Firstly, to take the groups that were given iron, we find that the haemoglobin concentration in the litters of Group I was lower than in the low-protein groups: the haemoglobin concentration in the Group I litters was on an average 8.7 g per 100 ml, whereas in the five litters of Group VII whose dams had been given iron it was 11.4 g per 100 ml—a significant difference. In Group I the haemoglobin concentration was least in the smallest litters (nos. 6 and 8) where the young were growing most rapidly. That the haemoglobin concentration was lower in the Group I litters than in the low-protein litters is also probably due to the more rapid growth of the former. Rapid growth implies that the supply of iron more readily becomes a limiting factor in haemoglobin formation. The amount of non-haemin iron in the liver per 100 g of fresh tissue is also significantly lower in Group I (mean 2.14 mg) than in the litters of Group VII whose dams were given iron (mean 7.91 mg). The explanation of these differences in non-haemin iron can also be sought in the different rates of growth.

In the Group II litters, where the dams received no iron supplement during pregnancy and lactation, the haemoglobin concentration was lower than in the litters of Group I (6.1 and 8.7 g per 100 ml respectively). The non-haemin iron in the livers was also significantly lower in Group II than in Group I (1.02 and 2.14 mg per 100 g of fresh tissue). Most of the litters of Group I had a certain amount of storage iron in the liver. The non-haemin iron in the carcase was also more in Group I, amounting to 1.15 mg per 100 g fresh tissue compared with 0.74 mg for Group II. In the litters of Group V, whose dams, like those of Group II, were given no iron supplement during the experiment, the figures for haemoglobin concentration and non-haemin iron in liver and carcase are almost as low as those in Group II. The females in Group II, and even more so in Group V, still had true storage iron in liver and carcase at the end of lactation. In spite of this the iron transfer from dam to litter was inadequate. The iron administered in the diet during pregnancy and lactation appears to be more readily transferred to the offspring than the storage iron of the dam.

The plasma iron values in the litters of Group I varied between relatively low levels (74 and 80 μg per 100 ml) and levels almost as high (175 and 198 μg per 100 ml) as those found in the experiment on growing animals in the groups on adequate iron and protein intake (see Chapter IV b). The values for non-haemin iron in liver

and carcase in two of the litters of Group I were as low as those for the litters of Group II. It would thus seem that on weaning the supply of iron readily becomes a limiting factor in haemoglobin formation, although it appears that iron deficiency is not the only cause of the low haemoglobin concentration in the litters, this being physiologically low on weaning. The haemoglobin concentration is low even in animals with relatively high plasma iron levels, and in which liver and carcase contain a certain amount of storage iron.

In the experiment on nutritional iron-deficiency anaemia (Chapter IV b) it was shown that the percentage decrease in mean corpuscular haemoglobin was as great as the percentage decrease in haemoglobin concentration, and that the percentage decrease in mean corpuscular volume was only slightly less. The haemoglobin concentrations in the litters of Group I were similar to those in the growing animals which received 22 % protein in the diet and 35 µg of iron per day (8.7 g and 8.4 g per 100 ml respectively). The mean corpuscular volume for the litters of Group I was on an average 58 cu. microns, and the mean corpuscular haemoglobin 17 micromicrograms: the corresponding values for the iron-deficiency anaemia group on 35 µg of iron per day were considerably lower—40 cu. microns and 12 micromicrograms respectively. The mean corpuscular Hb. concentration of the five litters of Group VII whose dams received iron during the experiment was on an average 33 % (31–35 %). In the Group I litters nos. 3, 6, and 8 the mean corpuscular Hb. concentration was low (27, 29, and 28 %), but in the other litters of the group it was just as high as in the aforementioned of Group VII which, owing to the plentiful supply of storage iron, could not have had iron-deficiency anaemia. The two litters of Group VII (nos. 42 and 44) whose dams received no iron had a lower haemoglobin concentration than the others in the group, and the mean corpuscular Hb. concentration was reduced (29 and 26 %).

Concerning haemoglobin formation in the litters it may be said *in summary* that the haemoglobin concentration is normally low on weaning (*c.* 10 g per 100 ml or slightly higher), and at the same time the risk of iron-deficiency anaemia is great.

CHAPTER VI

Haemoglobin Formation and Carcase Protein in Protein-Depleted Rats under Treatment with Cortisone or ACTH

In the protein-depleted rat I found a slightly lowered haemoglobin concentration and a marked diminution in the total haemoglobin in relation to the carcase protein. The weight of the adrenals fell parallel with the fall in total haemoglobin.

After adrenalectomy or hypophysectomy in animals the total haemoglobin diminishes more than the body-weight (Swingle, Vars, and Parkins, 1934; Garcia, Van Dyke, and Berlin, 1952). The following experiment in which protein-depleted male rats were treated with cortisone or ACTH was performed to determine whether these hormones have any effect upon haemoglobin formation during protein deficiency.

Experimental

Group I. Protein-deficient animals. Controls. Ten adult male rats weighing 288–342 g were placed on a diet containing 3.5 % of protein (spray-dried hen's egg-white). Each animal was offered 18 g of feed and 1 mg of iron per day, the whole ration being consumed. The animals were kept on the protein-deficient diet for 51–55 days, and the following determinations were then done for each animal: body-weight, haemoglobin concentration, total haemoglobin, total amount of protein in the carcase, plasma iron, and weight of adrenals.

Group II. Protein-deficient animals treated with cortisone. Eight male rats of the same age and weight as those in Group I were placed on the same protein-deficient diet. After 30 days the animals were given daily intramuscular injections of 1.5 mg of cortisone ("Cortone", Merck and Co.) per 100 g body-weight: the injections were made into the muscles of the thigh once daily for 21 days. The experiment was broken off 24 hours after the last cortisone injection, and estimations were carried out as in Group I.

Group III. Protein-deficient rats treated with ACTH. This group consisted of five male rats whose mean weight was slightly higher than that of the two preceding groups (332 g, cf. 314 g in Group I). The animals received the same low-protein diet as Groups I and II. After 30 days, administration of ACTH ("Cortico-Depot", Nyegaard & Co., Oslo) was begun, 3 mg per 100 g body-weight being injected into the thigh-muscles once daily for 21 days. Twenty-four hours after the last injection investigations were carried out as above.

At the end of the experiment the animals of Groups II and III had been on low-protein diet for exactly as long as the controls in Group I.

Results

The results are given in Tables 17 a and 17 b. The protein-deficient animals receiving cortisone diminished more in weight than the controls. The carcase protein in the former amounted to 30.7 g, and in the latter to 35.8 g. In contrast to the carcase protein the total haemoglobin in the cortisone-treated animals did not fall. In fact one of the animals had as much as 3.42 g of haemoglobin, the highest value I have ever found in a rat. Since the haemoglobin concentration in this animal was the same as in the others in the group, the blood volume must have been greatly increased. The quotient g Hb. per g carcase protein in the cortisone animals was 0.076, and in the untreated controls 0.056. The increase in this quotient in the cortisone animals is due chiefly to the reduction in carcase protein.

The conclusion which can be drawn from this experiment concerning the effect of cortisone on total haemoglobin in protein-depleted rats is that cortisone in a dose of 1.5 mg per 100 g body-weight per day for 21 days does not in any event reduce the total haemoglobin.

The haemoglobin concentration was slightly higher in the cortisone-treated animals (17.1 g per 100 ml) than in the controls (15.7 g per 100 ml). The plasma iron showed a clear increase to an average of 212 µg per 100 ml compared with 142 µg per 100 ml in the controls.

The adrenals became considerably smaller during the cortisone treatment, weighing in one case only 8.7 mg.

Summarizing the results of cortisone treatment of protein-depleted animals the following can be established. Cortisone treatment results in an increase in haemoglobin concentration and plasma iron which is approximately as great as the fall caused by the protein deficiency. The carcase protein is reduced but the total haemoglobin remains unchanged, the quotient g Hb. per g carcase protein thus being increased. The adrenals show a marked fall in weight.

The ACTH-treated animals exhibited similar changes to the cortisone animals. They diminished in weight and lost carcase protein. The total

Table 17 a. Haemoglobin concentration, total haemoglobin, protein in the carcase, plasma iron, and weight of adrenals, in protein-depleted male rats treated with ACTH or cortisone.

Rat No.	Body-weight at beginning of experiment, g	Decrease in body-weight, g	Hb. concentration, g per 100 ml	Plasma iron, µg per 100 ml	Total haemoglobin, g	Protein in carcase, g	g Hb. per g protein in the carcase	Weight of adrenals, mg
Group I. Protein-depleted male rats. Controls.								
1	312	24	16.1	103	2.26	36.9	0.061	29.8
2	320	25	15.1	150	2.17	38.9	0.056	29.1
3	294	11	15.3	174	2.14	33.9	0.063	29.0
4	288	16	15.5	178	1.81	32.0	0.057	31.9
5	342	37	15.9	152	1.84	40.0	0.046	30.3
6	327	19	15.6	145	1.86	35.4	0.053	28.7
7	317	45	15.8	119	2.01	33.5	0.060	26.5
8	313	13	15.9	156	2.18	37.6	0.058	26.5
9	311	28	16.1	107	1.84	34.2	0.054	28.7
10	314	32	16.1	137	1.97	35.5	0.055	29.8
Mean	314	25	15.7	142	2.01	35.8	0.056	29.0
Group II. Protein-depleted male rats treated with cortisone.								
11	330	48	17.1	156	2.08	35.3	0.059	23.4
12	294	53	17.3	177	2.21	28.8	0.082	14.3
13	289	28	17.1	208	2.33	28.9	0.080	18.4
14	330	76	17.1	222	2.10	31.6	0.067	17.0
15	308	59	16.8	246	2.00	28.5	0.070	15.9
16	320	66	16.7	208	2.01	31.2	0.065	8.7
17	318	61	17.9	237	2.17	29.9	0.073	18.8
18	342	72	16.4	240	3.42	31.3	0.109	19.6
Mean	316	58	17.1	212	2.29	30.7	0.076	17.0
Group III. Protein-depleted male rats treated with ACTH.								
19	324	36	17.1	205	2.10	32.5	0.065	84.4
20	321	47	15.5	176	2.27	29.9	0.076	84.9
21	340	62	16.3	202	2.20	33.7	0.065	96.8
22	351	59	16.5	192	2.21	34.3	0.064	92.5
23	324	59	15.5	201	2.14	31.8	0.067	104.8
Mean	332	53	16.2	195	2.18	32.4	0.067	92.7

haemoglobin seemed to be unchanged, and the quotient g Hb. per g carcase protein rose. The plasma iron increased on an average to 195 µg per 100 ml, but no increase in haemoglobin concentration could be demonstrated. The adrenals increased in weight up to 84.4–104.8 mg. After a few days the animals became noticeably sluggish, and it was clear that the dose was too high.

Table 17 b. Comparison between Groups I and II and between Groups I and III.

	Differences between Group I and Group II		Differences between Group I and Group III	
	D	*P*	*D*	*P*
Decrease in body-weight, g .	33	<0.001	28	<0.001
Protein in carcase, g	5.1	<0.001	3.4	<0.01
g Hb. per g protein in the carcase	0.020	<0.01	0.011	<0.01
Hb. concentration, g per 100 ml	1.4	<0.001	0.5	<0.2
Plasma iron, µg per 100 ml .	70	<0.001	53	<0.001
Weight of adrenals, mg . . .	12.0	<0.001	63.7	<0.001

Discussion

Clark (1953) has shown that the effect of cortisone on protein metabolism is more a restriction of protein synthesis than an increase in protein catabolism. This applies to the protein in the carcase. In contrast, Clark found that more protein was synthesized in the liver in cortisone-treated animals than in the controls. The serum protein concentration increased during treatment with cortisone. Since albumin is formed in the liver, Clark considers the increased serum protein to be a further indication of the accelerated protein anabolism in the liver. Roberts (1953) has shown the rate of liver regeneration to be proportional to the level of circulating adrenocortical hormones in the partially hepatectomized rat.

In the experiments described in Chapter III adult male rats on a protein-deficient diet showed a diminished percentage content of protein in the liver, the adrenals becoming at the same time markedly reduced in weight: the cortisone-treated animals of this experiment had large fatty livers.

Garcia, Van Dyke, Huff, Elmlinger, and Oda (1951) found that treatment of normal rats with ACTH (0.5 mg twice daily) for 116 days raised the total volume of circulating red cells to about 1.3 times that of normal, untreated controls. Normal animals which were given 1 mg of ACTH daily for 34 days showed no significant increase in total red cell volume. It would therefore have been interesting to treat protein-depleted animals with ACTH over an extended period and with a smaller dose to determine whether the total haemoglobin increases in protein-deficient animals on a long course of ACTH. For the same reason it would have been of interest to treat protein-depleted animals with cortisone for a longer period, perhaps here too using a smaller dose.

Cartwright, Hamilton, Gubler, Fellows, Ashenbrucker, and Wintrobe (1951) examined the effect of a single injection of ACTH or adrenocortical extract upon the plasma iron in normal dogs. The plasma iron fell, the minimum being reached 6–8 hours after the injection. The maximum percentage fall in plasma iron amounted on an average to 50 %, and after 24 hours the plasma iron had returned to its initial value or higher. The plasma iron was lower in adrenalectomized animals than in controls. These results too indicate a connection between adrenal function and plasma iron.

The diminution in adrenal weight in cortisone-treated animals is due to depression of the pituitary secretion of adrenocorticotrophic hormone by the cortisone (Ingle, 1938).

In short, it may be said that it has been proved that cortisone and ACTH do not have the same effect on protein metabolism in all organs in the normal rat. Whereas these hormones produce a diminution in the carcase protein, protein synthesis in the liver and haemoglobin formation are stimulated: in the protein-depleted rats of my experiments treated with cortisone for 3 weeks the carcase protein diminished by about 15 %, the total haemoglobin being unchanged and the haemoglobin concentration slightly raised. All these findings support the hypothesis put forward in this work, that the diminution in total haemoglobin in relation to the carcase protein, which takes place during protein deficiency, is secondary to the hormonal adaptation which results from the protein deficiency.

CHAPTER VII

Haemoglobin Formation and Adrenal Weight in Protein-Depleted Rats Bled by Cardiac Puncture

If protein-depleted animals are bled they are able to regenerate large quantities of blood. Haemoglobin synthesis then has priority over the carcase protein (Whipple *et al.*, 1947). This is the opposite of the state of affairs in non-bleeding low-protein animals, in which the total haemoglobin diminishes more than the carcase protein. In the preceding chapters experimental evidence has been put forward in support of my hypothesis that the diminution in total haemoglobin during protein deficiency is secondary to suppressed adrenal function caused by the protein deficiency. It is therefore of interest to study the weight of the adrenals in protein-deficient rats with vigorous regeneration of blood after bleeding.

Experimental

A series of male rats weighing about 300 g was placed on a diet containing 3.5 % of protein. One month later blood-letting by means of cardiac puncture was begun. Cardiac puncture was performed under ether anaesthesia. The heart was palpated, and a no. 14 needle thrust through the chest wall towards the heart. When the heart was felt against the point of the needle this was introduced a few more millimetres until blood escaped through the needle. Three millilitres were drawn up slowly into a syringe. The depth of anaesthesia was adjusted so that the animals regained consciousness more or less immediately after the procedure; they were not obviously shocked. In the beginning the cardiac punctures were quite often unsuccessful, but after a little practice it was usually possible to perform them without trouble. Four days after the last cardiac puncture the total haemoglobin, haemoglobin concentration, plasma iron, carcase protein, and weight of the adrenals were determined on each animal. No sign of bleeding into the pericardium could be seen.

Table 18 a. Haemoglobin lost on blood-letting, and haemoglobin concentration, total haemoglobin, protein in the carcase, and weight of adrenals, in protein-depleted male rats bled by cardiac puncture.

Rat No.	Body-weight at beginning of experiment, g	Decrease in body-weight, g	Hb. lost on blood-letting, g	Bleeding period, days	Hb. concentration, g per 100 ml	Plasma iron, µg per 100 ml	Total haemoglobin, g	Protein in carcase, g	Weight of adrenals, mg
1	322	30	1.34	14	9.2	130	1.63	36.3	37.0
2	328	38	1.29	13	14.1	161	1.83	35.8	33.6
3	306	79	1.37	21	11.3	120	1.08	29.1	33.8
4	330	77	2.19	25	11.6	52	0.97	32.0	36.1
5	294	55	1.51	15	13.3	84	1.36	30.4	32.1
6	327	43	1.83	15	12.2	169	1.43	35.6	36.0
7	318	60	2.31	23	12.8	61	1.69	30.2	43.0
Mean	318	55	1.69	18	12.1	111	1.43	32.8	35.9

Results

Tables 18 a and 18 b show the figures for seven males on which a series of successful cardiac punctures were carried out. The body-weights at the beginning of the experiment were approximately the same as for the low-protein animals which serve as controls (Table 17, Group I). Over a period varying from 14 to 25 days a quantity of blood containing on an average 1.69 g of haemoglobin (1.29–2.31 g) was let from each of the animals. At the end of the experiment the total haemoglobin was on an average 1.43 g (0.97–1.83 g): the low-protein controls had an average total haemoglobin value of 2.01 g (1.81–2.26 g). The bled animals thus synthesized approximately 1 g more of haemo-

Table 18 b. Comparison between the bled animals and the non-bled controls.

	Differences between the bled animals and the non-bled controls	
	D	*P*
Decrease in body-weight, g	30	<0.01
Protein in carcase, g	3.0	<0.05
Weight of adrenals, mg	6.9	<0.001

The animals in Group I (Table 17 a) are taken as controls.

globin than the controls during a period of 14–25 days. The life-span of the red blood cell in the male rat has been shown to be 68 days (Berlin *et al.*, 1951). Thus in protein-depleted rats with a total haemoglobin of 2.01 g the amount of haemoglobin synthesized per day should amount to 0.0295 g, provided that the life-span of the red cells is the same as in the normal rat. Synthesis of a further 1 g of haemoglobin in 14–25 days implies an increase in haemoglobin synthesis of 130–240 %.

The haemoglobin concentration varied between 9.2 and 14.1 g per 100 ml. There was no significant difference between the percentage decrease in haemoglobin concentration and the percentage decrease in total haemoglobin in the haemorrhagic anaemia. In three cases the plasma iron was lower than the values usually found in protein-depleted rats not bled.

As was to be expected, the cardiac-puncture animals diminished more in weight than the controls (55 g and 25 g respectively). The carcase protein of the cardiac-puncture animals was on an average 3 g less than that of the controls, the carcase thus having been drained of protein for use in the synthesis of haemoglobin and plasma proteins.

The adrenals increased in weight during the blood-letting period: they weighed on an average 35.9 mg in the animals bled compared with 29.0 mg in the controls, the difference being highly significant. Several animals which were subjected to repeated cardiac puncture without any blood being let showed no increase in adrenal weight. It can thus be taken for granted that the increase in weight of the adrenals was caused by the loss of blood.

Discussion

It is of course possible that the increase in weight of the adrenals in the cardiac-puncture animals has no influence on the regeneration of blood. On each occasion about 20 % of the blood-volume was removed, and such a large haemorrhage may well be expected to place the animal in a state of slight shock (as mentioned, the animals were not obviously shocked). The increase in adrenal weight may have been produced by haemorrhagic shock, and it is impossible to determine whether it is of any significance in the regeneration of blood in these blood-letting experiments. The experiments show, however, that in protein-deficient animals it is comparatively easy to stimulate the adrenals by the letting of blood. Anaemia alone has no effect upon the weight of the adrenals, as can be seen from the iron-deficiency experiments on growing animals (see Tables 8 b and 9 b).

In this connection I would draw attention to the weight of the adrenals in rats in which haemoglobin formation had been stimulated by means of low barometric pressure. Sundstroem and Michaels (1942) found

that the adrenals doubled in weight in rats exposed to a barometric pressure of 300 mm Hg., and nearly trebled at a pressure of 260 mm Hg. Again, it is possible that the stimulated haemoglobin synthesis and the increased adrenal weight are independent phenomena. Nor does this investigation by itself prove that the stimulated adrenal function is of any significance in the enhanced haemoglobin synthesis.

More direct support for the part played by the pituitary-adrenal system in achieving maximum haemoglobin synthesis is found in an investigation by Silbergleit (1952). He found that regeneration of blood was incomplete after haemorrhage in hypophysectomized animals. The haemoglobin concentration, haematocrit reading, and red cell count were determined, but not the total haemoglobin.

In summary, the incomplete regeneration of blood after haemorrhage in hypophysectomized animals, the simultaneous increase in total haemoglobin and adrenal weight at low barometric pressures, and the demonstration in this experiment that the letting of blood from protein-depleted animals causes the adrenals to increase in weight, all indicate that vigorous blood formation depends to a greater or lesser extent upon adrenal function.

CHAPTER VIII

Quantitative Studies on Storage Iron in Man

The Problem

In connection with my animal experiments I have performed some quantitative determinations of the storage iron in man, chiefly in organs obtained post mortem. My animal experiments demonstrate clearly the effect of changes in the total haemoglobin on the amount of iron in the stores. In earlier investigations on the storage iron in man due regard has not been paid to the state of the total haemoglobin. For example, Gross, Sandberg, and Holly (1942) have shown that the total amount of iron (haemin-bound + non-haemin) in liver and spleen is increased in patients with malignant tumours. They find it remarkable that this is also the case in tumour patients without anaemia. Great changes in the total haemoglobin are, however, possible without the haemoglobin concentration being affected to any considerable degree, as for example in protein deficiency in the rat. In patients with advanced, metastasizing tumours there is deficiency of protein in the organism: they take little nourishment, and the growth claims material for the synthesis of tumour protein.

Clark *et al.* (1947) estimated the total haemoglobin in 89 patients with a history of loss of weight, 41 of whom had malignant tumours. The total haemoglobin was calculated from the haematocrit reading and the plasma volume determined with T-1824. The total haemoglobin in the tumour patients amounted to only 50 % of the standard value calculated for their ordinary weight. The blood volume was 72 % of the corresponding standard blood volume. Clark *et al.* introduced the term "chronic shock" for patients with similarly reduced blood volume. Wilson and Hardy (1953), too, investigated the plasma volume and red cell volume in malnourished cancer patients with the aid of T-1824. They found low red cell volumes with normal or increased plasma volumes. Although the blood volumes were adequate when calculated on the basis of these patients' actual weights, they were low on the basis of their normal weights.

In patients dying of tumour cachexia the total haemoglobin may thus be taken to be diminished.

Gross *et al.* determined the total iron (including haemoglobin iron, enzyme iron, and non-haemin iron) in the organs. I considered it to be of interest to determine the non-haemin iron alone in cases of malignancy, firstly to find out whether the non-haemin iron is increased in patients with malignant tumours, and, secondly, to determine whether this increase is no greater than can be explained by a reasonable decrease in the total haemoglobin.

As a rule the liver, spleen, and bone-marrow are taken to be the organs which contain storage iron. Kooyman (1949), however, has shown that heart muscle, psoas, and the kidneys sometimes contain more non-haemin iron than the liver and spleen. No quantitative chemical determinations of the non-haemin iron in bone-marrow have ever been carried out. I have therefore estimated the non-haemin iron in bone-marrow from rib. In studying the distribution of the storage iron among different organs I have paid particular attention to the problem of whether deficiency of storage iron in one storage organ, e.g. bone-marrow, is accompanied by deficiency in other organs. In living subjects it is usually convenient only to take samples from a single storage organ, viz. the bone-marrow by means of sternal puncture.

Since no quantitative microchemical determinations of the storage iron in bone-marrow in idiopathic hypochromic anaemia have been done, I have determined the non-haemin iron in sternal marrow (sternal puncture) in a few cases of this kind. My object has been to determine how low the values for non-haemin iron in the bone-marrow are in idiopathic hypochromic anaemia. It is valuable to be able to compare these values with the ones I found at autopsy in the bone-marrow of patients with chronic haemorrhage, in whom the storage iron was exhausted. It is also interesting to compare the amounts of iron found in human subjects with exhausted iron stores with those in rats suffering from nutritional iron-deficiency anaemia. In assessing the figures for storage iron it is essential to know the organ's content of non-haemin iron after all storage iron has been used up. Other more variable comparative values are the iron quantities found in normal human subjects.

Rath and Finch (1948) examined histochemically the storage iron in fragments of bone-marrow obtained on sternal puncture. Sixteen patients with iron-deficiency anaemia exhibited no haemosiderin, and seven of these had no haemorrhage. In infections, pernicious anaemia, haemochromatosis, and after multiple blood transfusions, much haemosiderin was seen. Patients with hepatic cirrhosis and nephritis had varying amounts of haemosiderin owing to varying degrees of haemorrhage. Davidson and Jennison (1952) similarly investigated the incidence of iron in the bone-marrow with the aid of Prussian blue in 165 patients. In 30 of 32 cases of hypochromic anaemia no iron was demonstrable in the marrow, and the serum iron was low. In cases

of anaemia secondary to sepsis or chronic uraemia the bone-marrow iron was increased but the serum iron was low. The greatest deposition of haemosiderin in the marrow was associated with repeated blood transfusions.

Schairer and Rechenberger (1948) examined the correlation between the histochemically and microchemically determinable iron in liver and spleen. In general these methods gave similar results, but exceptions occurred in isolated cases. When the liver contained no histochemically demonstrable iron its content of non-haemin iron was 12.0 mg per 100 g of fresh tissue; the corresponding figure for the spleen was 15.1 mg per 100 g of fresh tissue. Large quantities of stored iron may thus be present in liver and spleen without being histochemically demonstrable.

One of the greatest difficulties in investigations on storage iron in man is to obtain a normal series. Schairer and Rechenberger (1948) examined the storage iron in liver and spleen in 18 men of various ages, of whom 16 had been killed accidently, one had died of spontaneous pneumothorax, and one had committed suicide. The iron in the liver was determined as total iron after washing out the blood by perfusion with physiological saline. The splenic iron was determined after extraction of the haemoglobin from the homogenized specimen with the aid of physiological saline. The average figure for the liver was 28.5 mg per 100 g of fresh tissue (8.4–60 mg), and for the spleen 30.0 mg (22.8–60 mg). Schairer and Rechenberger found the quantity of storage iron to increase with age: In their youngest ten cases, aged 14–30 years, the average value for storage iron in the liver was 20.1 mg per 100 g of fresh tissue (8.4–50 mg), and in the eight remaining cases, aged 44–82 years, 39.1 mg (22.8–60 mg); the corresponding figures for the spleen were 21.4 mg (9.5–39.6 mg) and 54.4 mg (10–117 mg). This work was done in Germany during the Second World War, and it may be that the results were influenced by malnutrition. Clark *et al.* showed that the total haemoglobin in seven cases of malnutrition was reduced to 53 % of the standard value for the patients' normal weights. In war-time, when food-supplies are limited, the older members of the community are commonly the worst fed.

Brückmann and Zondek (1930) determined the non-haemin iron in liver and or kidneys from 21 autopsies on adults aged 16–84 years; six of these can be said to have been normal cases, five having been killed and one having committed suicide. The amount of iron is expressed in mg per kg dried tissue, the percentage content of water in the organ being given in every case. I have recalculated their figures to mg of iron per 100 g of fresh tissue in order better to compare them with my own and those of Schairer and Rechenberger: a 24-year-old woman who died of suffocation had 14.0 mg of iron per 100 g of liver tissue; three men aged 30, 36, and 44, who had been shot or hanged,

had 17.2, 20.6, and 13.9 mg per 100 g of fresh liver tissue respectively; and a 34-year-old man who died of barbiturate poisoning had 30.1 mg of iron per 100 g of fresh liver tissue.

Own Investigations

Experimental details. Only subjects examined a short time after death (not more than 24 hours) were used for determining the content of non-haemin iron. Bone-marrow was obtained from the ribs; these were cut across and the marrow pressed out. Several pieces were taken from each of the other organs concerned in order to obtain as representative a specimen as possible from the organ. Psoas was used throughout for determining the non-haemin iron of muscle. The homogenization was done by hand with glass knives in a mortar.

The Non-haemin Iron in Bone-Marrow, Liver, and Spleen

"Normal" cases. To get some idea of the amount of non-haemin iron present normally I have done estimations in three subjects who died from accidental causes (Group I, Table 20). I further determined the non-haemin iron in eight biopsy specimens taken from the livers of patients undergoing upper abdominal operations, in all cases but one for ulcer or gall-bladder diseases. I have attempted to select patients in good state of nutrition and not known to have had haemorrhage. The results are given in Table 19.

The liver and bone-marrow of the two young men killed in motor accidents (Table 20) contained 15–20 mg of iron, and the spleen about 10 mg per 100 g fresh tissue. A 51-year-old farmer killed by a horse-kick had about 3 mg of non-haemin iron per 100 g fresh tissue in liver, bone-marrow, and spleen. Although the iron content of liver, bone-marrow, and spleen was considerably lower in this case, the non-haemin iron in heart, kidneys, lungs, and psoas was approximately the same in amount as in the two young men. This tallies with my findings in the rat, that the storage iron in liver, bone-marrow, and spleen is utilized before that of the carcase. In these three "normal" cases the heart muscle contained 1.4–1.9 mg, the lungs 1.4–2.3 mg, the kidneys 1.2–1.8 mg, and the psoas 0.6–0.9 mg of iron per 100 g fresh tissue.

The first three operation cases were men aged 35–48 years suffering from peptic ulcer without known haemorrhage in the period before operation, and in good general condition. The fourth was a 48-year-old man with acute cholecystitis. In these four cases the liver iron varied between 15.6 and 22.8 mg per 100 g fresh tissue. The fifth case was a man of 36 years with duodenal ulcer, who, on account of falling haemoglobin level, must be suspected to have had haemorrhage in the

Table 19. Non-haemin iron in biopsy-specimens taken from livers of patients undergoing upper abdominal operations

Case No., age	Sex	Diagnosis	Hb. conc., g per 100 ml	Colour index	Serum iron, µg per 100 ml	Non-haemin iron in liver, mg per 100 g fresh tissue	
1. H.S., 42	M	Peptic ulcer	14·2		165	16·9	
2. E.A., 35	M	„ „	13·7		124	22·8	
3. B.L., 42	M	„ „	13·2	0·95	140	19·8	
4. G.E., 48	M	Acute cholecystitis with calculus + acute pancreatitis	14·9	0·87	80	15·6	
5. O.F., 36	M	Peptic ulcer	14·4	0·87	197	16·4	
6. V.C., 55	M	Carcinoma stomach + acute polyarthritis + tuberculous cervical lymphadenitis	6·6	0·81	40	8·9	Blood transfusions 3 × 400 ml
7. M.A., 52	F	Cholelithiasis	12·8	0·93		12·9	
8. G.J., 68	F	Cholecystitis with calculus + choledocholithiasis + thoracic kyphoscoliosis	10·3	0·72	76	9·7	

period immediately before operation the liver contained 10·1 mg of iron per 100 g of fresh tissue. Case no. 6 was a man aged 55 suffering from carcinoma of the stomach, acute polyarthritis and tuberculous cervical lymphadenitis. He had evidently bled from his carcinoma and had severe anaemia. Before operation he was given 2 × 400 ml of blood.] The liver contained 8·9 mg of non-haemin iron per 100 g of fresh tissue. Case no. 7 was a woman of 52 with cholelithiasis but otherwise healthy, and she had 12·9 mg of iron per 100 g of liver tissue. The last case was also a woman aged 60 with cholelithiasis and hypochromic anaemia, whose liver contained 9·7 mg of iron per 100 g fresh tissue.

The first two accident cases and the first four operation cases suggest that in normal men with loaded iron stores the liver contains about 15–20 mg of non-haemin iron per 100 g of fresh tissue. In the two young men accidents killed the bone-marrow contained approximately as much iron as the liver. The quantities of storage iron which I found in the liver in my "normal" cases are of the same order as the normal values of Brinkmann and Zondek and the mean for the younger men normal cases of Schairer and Rechenberger.

Malignant tumours and leukaemia (Group II, Table 20). This group comprises 18 cases of malignant tumours and two of leukaemia. The cases have been divided into two sub-groups, those with known haemorrhage and those without.

In the sub-group with haemorrhage the storage iron was low. In the first two cases the bone-marrow contained only about 1 mg of non-haemin iron per 100 g fresh tissue. In the first case the liver contained 3·5 mg of iron per 100 g of fresh tissue, and in the second 1·9 mg of iron. Brinkmann and Zondek reported in their series a case of carcinoma of the stomach with severe anaemia in which the liver contained 1·4 mg of iron per 100 g of fresh tissue.

In the first five non-haemorrhage cases the non-haemin iron values were high in at least one of the storage organs. In the sixth case, myeloid leukaemia, the non-haemin iron content of the bone-marrow was very high and that of the spleen fairly high. [This patient, however, had received about 2·4 litres of blood during the last two months of life.] The tenth case of this sub-group (case no. 19, Table 20), one of cerebral tumour, also had high non-haemin iron values for bone-marrow and liver. In the remaining non-haemorrhage cases the non-haemin iron levels were lower than the ones just mentioned. For example, case no. 20, a woman with cerebral tumour, had only 2 mg of iron per 100 g fresh tissue in bone marrow, 1·4 mg in liver, and 1·6 mg in spleen.

In none of the tumour cases was the quantity of storage iron so great that the increase cannot be explained by a fall in the total haemoglobin of the magnitude which, according to Clark *et al.*, is usual in cases of

Table 20. Non-haemin iron in various human organs (autopsy).

Case No., age	Sex	Diagnosis	Hb. conc., g per 100 ml	Colour index	Non-haemin iron, mg per 100 g fresh tissue								Remarks
					Bone-marrow	Liver	Spleen	Kidney	Lungs	Muscle (psoas)	Heart	Brain	
		Group I. Accidents.											
1. G.B., 27	M	Motor accident			20.7	16.0	8.3	1.2	1.4	0.7	1.6	4.1	
2. H.N., 30	M	Motor accident			15.2	18.5	11.7	1.8	2.1	0.8	1.9		
3. G.K., 51	M	Killed by horse kick			3.9	3.0	2.3	1.6	2.3	0.9	1.4	2.5	
		Group II. Malignant tumours and leukaemia, (a) haemorrhagic.											
4. D.G., 58	M	Ca stomach (operation)	9.3	0.69	0.9	3.5	1.9	1.3	1.6		1.9	5.6	Blood 1200 ml
5. J.P., 74	F	Ca stomach + mitral stenosis	6.1	0.60	1.1	1.9	2.4	0.7	7.6	0.3	0.3		
6. H.E., 55	M	Sarcoma small intestine	7.5	0.84	6.0	2.6	5.0	2.1	2.4	1.2	2.3		Iron per os
7. R.J., 41	M	Ca stomach	7.3	0.63	13.3	4.9	19.6	0.5	1.6	0.2	1.4		
8. S.L., 66	F	Ca pancreas	11.9	1.00	5.7	3.3	2.9	0	0		2.1	2.4	
9. N.T., 43	M	Ca bronchus	13.5	1.15	3.6	4.6	4.5						Haemoptysis
		(b) non-haemorrhagic.											
10. K.E., 70	M	Ca prostate			28.0	47.2	24.5	0	0.2	1.6	1.8	4.3	
11. J.R., 76	M	Ca prostate + chronic pyelonephritis	7.7	1.05	25.7	18.2	65.6	1.1	16.4	1.4	2.2	4.3	
12. M.L., 51	F	Ca thyroid	10.4	0.91	50.2	10.7	17.7	0	0	0	1.3	2.4	
13. O.H., 49	M	Secondary carcinoma of bone	9.6	0.84	18.2	26.0	32.2	0.1		0.4	0		
14. E.S., 45	F	Ca breast	9.0	0.82	25.9	9.7	23.7	0	0	1.3	0.7	4.6	Serum Fe 100
15. E.E., 66	F	Myeloid leukaemia + diabetes mellitus	6.6	1.02	65.2	17.5	25.6	3.0	1.5	0.9	2.6		Blood 2400 ml
16. J.Å., 63	F	Lymphosarcomatosis + pulmonary embolus	12.9	1.00	9.8	6.7	14.0	1.5	8.1	1.6	2.2		Tumour 3.2
17. T.A., 43	M	Polycythaemia vera + Osteosclerosis	7.3	0.90	6.3	18.9	31.1	4.4	1.2	1.4	0.8	3.4	Serum Fe 65
18. G.N., 46	M	Reticulum cell sarcoma	11.7	0.78	24.6	8.9	5.2	2.0	2.4	1.3	2.9	2.2	
19. H.S., 44	M	Cerebral tumour (glioblastoma)	14.2	1.10	26.5	29.7	12.6	0	3.3	1.5	2.0	5.2	Tumour 4.7
20. M.H., 41	F	Cerebral tumour	12.4	0.96	2.0	1.4	1.6					4.2	
21. G.J., 76	M	Lymphatic leukaemia + hepatic cirrhosis + fracture of 10th thoracic vertebra (accident)	12.9	0.98	5.6	5.1	9.4	1.1	3.2	1.3	1.4		
22. S.B., 51	F	Ca breast	12.5	0.87	15.3	7.1	21.6						
23. R.O., 52	F	Ca bronchus	10.0	0.93	10.8	5.1	10.4						

Group III. Infections.																
24. J.L.,	50	M	Sub-acute bacterial endocarditis	7.8	0.91	21.8	47.2	55.6	5.4	4.3	2.5	2.7		
25. E.M.,	55	F	Same	5.0	0.75	6.0	5.5	11.6						
26. G.A.,	31	M	Chronic pancreatitis	9.3	0.92		37.0	87.2						Serum Fe 74 Blood
27. J.F.,	78	M	Choledocholithiasis + acute cholangitis	9.9	0.80	31.9	17.1	11.2	3.0	1.8	1.0	1.0		3600 ml
28. S.A.,	48	M	Acute pancreatitis	13.3	0.87	17.6	17.0	26.1	1.2	1.5	1.2	1.4	2.3	
29. E.E.,	56	F	Chronic rheumatoid arthritis + chronic nephritis	6.0	0.85	7.5	17.8	29.6	0	0	1.7	1.8	5.2	
30. K.A.,	49	M	Pulmonary tuberculosis + tuberculous peritonitis + acute miliary tuberculosis of the liver	8.6	0.88	11.9	23.6	13.2						
31. V.P.,	65	F	Renal tuberculosis + agenesis of rt. kidney + Diab. mellitus	9.8	0.67	12.2	6.2	7.1	2.8	1.3	1.3	2.5	2.5	
32. G.B.,	26	M	Acute miliary pulmonary tuberculosis + tuberculous meningitis	13.3	0.93	16.6	14.3	22.6						
33. E.H.,	47	M	Fracture of tibia + fat emboli + pulmonary tuberculosis	11.6	0.90	10.2	15.7	15.9	2.7	3.7	1.9		5.0	
Group IV. Miscellaneous.																
34. K.L.,	67	F	Acrestic anaemia + mitral stenosis + cerebral embolus	7.7		28.7	37.2	43.6	3.5					
35. N.G.,	38	M	Aplastic anaemia	3.2	1.46	73.4	36.1	27.9	3.2	16.6	1.5	3.1	3.8	Blood 9000 ml
36. S.A.,	66	F	Vascular encephalopathy + anaemia of uncertain origin	7.3	0.75	1.5	1.2	2.1						
37. A.B.,	80	F	Cerebral haemorrhage	15.9	0.95	4.5	6.0	5.0	2.0	4.6	0.8	1.6	3.8	
38. M.S.,	55	F	Coronary occlusion	13.5	1.06	9.1	4.1	8.7	2.1	1.9	0.8	0.7	4.9	
39. S.L.,	74	F	Bronchiectasis + renal amyloidosis + uraemia	9.8	0.91	5.7	10.5	13.1	1.2	7.1	3.0	1.6		Serum Fe 22
40. J.E.,	78	M	Bronchiectasis + bronchopneumonia + coronary arteriosclerosis + hyperthyroidism	9.8	1.00	6.8	2.1	8.2	1.7					Serum Fe 56
41. B.F.,	50	M	Incompensated valvular disease of the heart	12.9	0.98	2.3	0.1	2.1	1.6	5.6	0.7	0.7		
42. L.T.,	54	F	Same			4.6	6.1	4.5	1.7	5.9	0.6	0.7		

malignant tumour. In four of the first five non-haemorrhagic cases the haemoglobin concentration was known. Here the anaemia was so pronounced that, even without any fall in blood volume, there must have been a considerable fall in the total haemoglobin.

In calculating the approximate iron content of the bone-marrow I have taken the active marrow to weigh about as much as the liver. The liver and spleen were weighed in every case. Mechanik (1926) found the bone-marrow in the adult to weigh between 1.6 and 3.7 kg (average 2.6 kg), and that half of this consisted of red marrow.

Infections (Group III, Table 20). This group comprises ten cases. On the whole the iron stores were well charged. In seven cases the haemoglobin concentration was less than 10 g per 100 ml, the anaemia in no case being due to a shortage of storage iron. In the first case in this group, one of sub-acute bacterial endocarditis, there were large supplies of iron in the liver and spleen. The duration of the disease was about three months, during which time the haemoglobin concentration fell to 7.8 g per 100 ml and the patient lost about 12 kg in weight. The increase in non-haemin iron in liver and spleen can probably be explained by the fall in total haemoglobin. The iron stores in liver and spleen were also large in the third case in this group, but this patient had had blood transfusions containing altogether about 1.8 g of iron.

Clark *et al.*, in the investigation quoted above, also determined the total haemoglobin and blood volume in 28 cases of chronic infection with loss of weight. The total haemoglobin was reduced to an average of 59 % of the standard calculated for the normal weights of these patients, and the blood volume amounted to 76 % of the corresponding standard value.

Brückmann and Zondek found that the non-haemin iron of the adults in their series who died of disease was 20 % higher than in the healthy subjects who had been killed, but they could not offer any explanation for this. Their series, however, consisted of only 12 adults who died of disease. Whether an increase or decrease in storage iron is found in a series of autopsies depends upon the character of the diseases of which the patients died. If the total haemoglobin falls considerably in a relatively short time in the absence of haemorrhage, an increase in the iron stores follows. This is usual in many diseases such as malignant tumours, infections, and diseases associated with malnutrition.

Schairer and Rechenberger (1948) are of the opinion that the iron stores in the liver are not affected by diseases such as infections, tumours, and cerebral affections. It must be questioned whether this conclusion cannot be explained by the high "normal" values which the authors give for the high age-group with the greatest mortality from e.g. tumours. In many infections, such as sepsis, pneumonia, and tuberculosis, Schairer and Rechenberger found an increase in splenic iron which they believed to be due to increased haemolysis.

Miscellaneous (Group IV, Table 20). The first of the nine cases of this group was a 67-year-old woman with acrestic anaemia and large stores of iron. The second was a man aged 38 with aplastic anaemia, who had received blood transfusions containing altogether *c.* 4.4 g of iron. The non-haemin iron in liver, spleen, and lungs amounted in this patient to 1.2 g; the concentration of iron in the bone-marrow was high, but it was impossible to make a guess at its total amount since it was not known to what extent the weight of the bone-marrow was affected by the disease process. The iron content of the lungs was very high in this case (16 mg of non-haemin iron per 100 g of fresh tissue). The patient had for a long time suffered from pulmonary congestion with severe dyspnoea, and it is possible that he lost iron through the lungs.

With the exception of the first two cases, the non-haemin iron levels in liver, bone-marrow, and spleen were low in this group. In a 66-year-old woman with the ante-mortem diagnosis "vascular encephalopathy + anaemia of uncertain origin" (case no. 36) the non-haemin iron values were so low that it may be assumed that the iron stores were exhausted; the colour index was low, and the anaemia was in all probability due to iron deficiency. A woman of 80 years with cerebral haemorrhage (case no. 37) might well be considered a "normal" case: the non-haemin iron levels in bone-marrow, liver, and spleen were comparatively low (5–6 mg per 100 g of fresh tissue). A woman of 55 years who died of coronary occlusion (case no. 38) also had comparatively low iron stores, especially in the liver (4.1 mg per 100 g of fresh tissue).

Schairer and Rechenberger (1948) found that the iron stores are smaller in women than in men. The average non-haemin iron content of the liver in 112 men who died of various diseases was 397 mg, and the corresponding figure for 81 women was 224 mg. These authors do not give the distribution of the various diseases among their male and female series. In assessing the non-haemin iron in a pathological series of this kind account must be taken of changes in the total haemoglobin. The blood volume is greater in men, and the fall in total haemoglobin, which in many cases accompanies the ante-mortem decline, may also have been greater in the men of the series. The difference between the men and the women was, however, so great that this can hardly have been the only cause. The finding of comparatively low iron stores in some of my female cases also suggests that the iron stores are smaller in women than in men.

The Non-haemin Iron in Kidneys, Lungs, Muscles, and Brain

The kidneys. The non-haemin iron in the kidneys of 32 of the cases examined varied between 0 and 4.4 mg per 100 g of fresh tissue (Table

20); a further case (no. 24, with sub-acute bacterial endocarditis) had as much as 5.4 mg per 100 g fresh tissue, but here there were septic emboli in the kidneys which may have influenced the result. In six cases no non-haemin iron could be demonstrated in the kidneys. The liver, bone-marrow, and spleen in these cases contained varying amounts of storage iron, but in no case were the values extremely low. The higher levels (*c.* 3 mg per 100 g fresh tissue and over) for non-haemin iron in the kidneys were chiefly found in cases where the supply of iron in bone-marrow, liver, and spleen was abundant, but there appears to be no close correlation between the non-haemin iron of the kidneys and that of the bone-marrow, liver, and spleen.

Brückmann and Zondek found the non-haemin iron of the kidneys of 22 adults to vary between 1.1 and 5 mg of iron per 100 g of fresh tissue.

In summary it may be said that in man the kidneys do not contain large quantities of storage iron, but are insignificant as storage organs.

The lungs. These were examined for non-haemin iron in 30 cases, the values being found to vary between 0 and 16.6 mg per 100 g of fresh tissue. All cases where the amount of iron was 5 mg per 100 g or more were patients with pulmonary congestion. In one case of cardiac valvular disease (case no. 41) there was no storage iron in the liver, bone-marrow, or spleen, but the lungs contained 5.6 mg of non-haemin iron per 100 g of fresh tissue.

Rechenberger and Schairer (1943) found the quantity of iron to be increased in pulmonary congestion. The iron was determined after ashing, the haemoglobin previously having been washed out with physiological saline. Their normal cases had on an average as much as 11.7 mg of iron per 100 g of lung tissue. Seven cases of chronic heart disease with pulmonary congestion had an average of 65.8 mg of iron per 100 g tissue. Rechenberger and Schairer found that the iron in the liver and spleen diminished as the iron in the lungs increased, the iron in the lungs, however, being much more than that which disappeared from the liver and spleen. They assumed, therefore, that some of the iron had been absorbed from food and transferred to the lungs as serum iron.

The largest amount of non-haemin iron which I have found in the lungs amounted to 226 mg. In no case have my iron values been so high that it has been necessary to assume increased absorption, but it is clear that during congestion the lungs may contain considerably more non-haemin iron than, for example, the liver. It is not known to what extent the iron deposited in the lungs during congestion can be utilized in haemoglobin synthesis.

The muscle. The non-haemin iron was determined in the *psoas* in 29 cases, and I obtained values between 0 and 3.0 mg per 100 g fresh tissue (in 22 of the cases the figures were between 0.5 and 1.8 mg per 100 g). It is impossible to find any correlation between the concentra-

tion of non-haemin iron in the liver and psoas in the cases examined, but it would be interesting to estimate the non-haemin iron in muscle in more cases where the storage iron in liver, spleen, and bone-marrow is exhausted. By this means it would be possible to determine whether the non-haemin iron of muscle diminishes after that of the liver, bone-marrow, and spleen has been used up.

The concentration of non-haemin iron in muscle is comparatively low, but the total amount is quite considerable: if the muscles contain an average of 1 mg of iron per 100 g of tissue, and weigh altogether 25 kg, their total content of non-haemin iron would be 250 mg. In calculating the total amount of storage iron in the body it is essential to know to what extent the non-haemin iron of muscle can serve as storage iron.

In 30 cases I found the *heart muscle* to contain between 0 and 3.1 mg of non-haemin iron per 100 g of fresh tissue, in 25 of them the concentration lying between 0.7 and 2.6 mg per 100 g. The mean value for heart muscle was slightly higher than for psoas. No correlation between the storage iron in the liver and the non-haemin iron in the heart muscle could be found.

Schapira and Dreyfus (1948) estimated the non-haemin iron in muscle as the difference between total iron and haemin iron; using pyrophosphate they were able to extract only 60 % of the non-haemin iron. They examined several species of animals and different groups of muscles. In the skeletal muscle of the dog they found, using pyrophosphate, 3.8–5.6 mg of iron per 100 g dry substance, and in the heart muscle 8.9 mg per 100 g dry substance. Their figures for the ox were similar. The water content of the muscle is about 75 %. My figures for non-haemin iron in human muscle thus tally with those found by Schapira and Dreyfus for dog and ox.

Kooyman (1949) extracted haemin iron from the tissues as acid haematin, and determined the total iron in the remainder. The values for non-haemin iron in psoas in three adults were as high as 11.7, 30, and 98 mg per 100 g dry weight. In the last two cases he also determined the non-haemin iron in the heart muscle, these values being lower -23 and 11 mg per 100 g dry substance respectively; in a 66-year-old man he found in the heart as much as 34 mg per 100 g dry substance. Kooyman found similar large quantities of non-haemin iron in the heart muscle and psoas of a few children who died of various diseases. Some of Kooyman's figures are so high that they must be due to technical errors. In the kidneys, too, Kooyman found remarkably large amounts of non-haemin iron.

The brain. A specimen consisting of similar amounts of white and grey substance was taken from one of the frontal lobes, and homogenized in the ordinary way. In all, 19 cases were examined, the non-haemin iron being found to vary between 2.2 and 5.6 mg per 100 g of fresh

tissue (mean 3.83 mg per 100 g). The quantity of non-haemin iron in the brain appears to be independent of that in the other organs. In case no. 4, one of carcinoma of the stomach with very small quantities of non-haemin iron in the bone-marrow, liver, and spleen, the figure for the specimen of brain tissue examined was as high as 5.6 mg per 100 g of fresh tissue.

Tingey (1937) estimated the non-haemin iron in different parts of the brain. The iron was extracted with sodium pyrophosphate during 24 hours. He found in the cerebral cortex an average of 2.81 mg of non-haemin iron per 100 g of fresh tissue ($n = 7$, $\sigma = 0.63$), and in the white matter 2.59 mg ($n = 6$, $\sigma = 0.76$). The cerebellar cortex contained 2.83 mg ($n = 5$, $\sigma = 0.79$), the corpus striatum 9.92 mg ($n = 8$, $\sigma = 2.14$), the pallidum 22.1 mg ($n = 4$, $\sigma = 9$), the substantia nigra 26.8 mg ($n = 1$), and the thalamus 5.96 mg ($n = 3$, $\sigma = 0.68$) non-haemin iron per 100 g of fresh tissue. Tingey also determined the total iron in the brain after washing out the blood: the cortex contained 3.62 mg of iron ($n = 19$, $\sigma = 0.62$), and the white matter 3.77 mg of iron ($n = 17$, $\sigma = 0.48$) per 100 g of fresh tissue. The figures were lower in children than in adults: for example, the cerebrum in the newborn contained only 0.81 mg extractable iron and 0.00 mg total iron per 100 g of fresh tissue. In patients with general paralysis both the extractable and total iron of the cortex were increased.

Tompsett (1935), too, determined with the aid of pyrophosphate the extractable iron of the brain. His extraction time was only 15 minutes, and his figures were consequently too low: he obtained only 0.4–1.5 mg of iron per 100 g of fresh brain tissue.

Tingey's figures for total iron in the brain are similar to the amounts of iron which I was able to extract with 5 N HCl. A brain weighing 1400 g and containing 3.83 mg of non-haemin iron per 100 g fresh tissue (mean of 19 cases in my series) should contain a total of about 54 mg of non-haemin iron. This figure represents a minimum value, since the concentration of iron is greater in the basal ganglia. The physiological significance of the non-haemin iron of the brain is unknown.

Some General Remarks on the Storage Iron in Man

The determinations of non-haemin iron in different organs of man have shown that the bone-marrow, liver, and spleen are the most important iron-storage organs in the body. The total amount of non-haemin iron in the muscles seems to be approximately the same as that in the liver. In this investigation it has been impossible to determine whether the non-haemin iron of muscle can be utilized in haemoglobin synthesis, but if this should be the case it is probable that it is not drawn upon until the stores in the bone-marrow, liver, and spleen are ex-

hausted: in the iron-deficiency experiments on the rat considerable quantities of non-haemin iron were mobilized from the carcase after the stores in the liver, bone-marrow, and spleen had been used up. Roth, Jasiński, and v. Bidder (1951) estimated the total iron of the muscles in man after washing out the haemoglobin, and found it to be reduced in iron deficiency; the fall in iron concentration was so great that the myoglobin concentration must have been reduced.

In pulmonary congestion the lungs contain large quantities of non-haemin iron, but it is not known whether this iron can serve as storage iron.

A rough estimate of the total amount of non-haemin iron in the body of an adult man with loaded iron stores suggests that it amounts to about 1 g (*c.* 300 mg in liver and bone-marrow each, 250 mg in the muscles, and 100 mg in the spleen, kidneys, and lungs together). By repeatedly bleeding healthy men during 3–4 months, Haskins, Stevens, Finch, and Finch (1952) succeeded in mobilizing 1200–1300 mg of iron from the stores. These high figures, compared with the amount of storage iron which I found in the liver, bone-marrow, and spleen, make it appear that the non-haemin iron of the carcase in man, as in the rat, can be utilized for haemoglobin synthesis when necessary. It should be added that the figures of Haskins *et al.* are only approximate, since it is impossible precisely to determine the quantity of iron absorbed from the food. (Haskins *et al.* calculated from their earlier observations that about 3 mg of iron was absorbed per day in their experiments.)

In my investigation it has been possible to determine how much non-haemin iron is present in the bone-marrow, liver, and spleen after all storage iron has been used up. The lowest post-mortem values for non-haemin iron in the bone-marrow which I have found are 0.9, 1.1, 1.5, 2.0, and 2.3 mg per 100 g fresh tissue; the lowest values for the liver are 0.1, 1.2, 1.4, 1.9, and 2.1 mg per 100 g fresh tissue (the liver which contained only 0.1 mg was the seat of pronounced cardiac cirrhosis); and the lowest values for the spleen are 1.6, 1.9, 2.1, 2.1, and 2.4 mg of non-haemin iron per 100 g of fresh tissue. Some of these figures originated from cancer patients who had been drained of blood over a long period, and they may be taken to represent approximate minimum values for non-haemin iron in the organs in question. When the storage iron is exhausted the bone-marrow and liver thus contain 1–1.5 mg of non-haemin iron per 100 g of fresh tissue, and the spleen 1.5–2 mg. In the rat, the liver contained 0.9 mg of non-haemin iron per 100 g of fresh tissue when all storage iron had been used up.

Bone-marrow and liver together contain the greater part of the storage iron of the body, and it is this iron, together with that of the spleen, which is most readily mobilized at need. Knowledge of the storage iron content of the bone-marrow and liver gives a good idea of the total storage iron content of the body. Since in living subjects

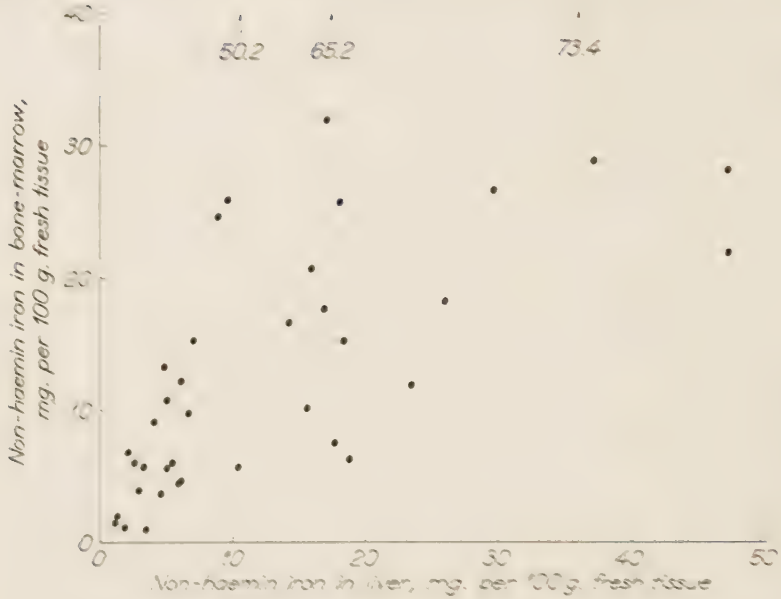

Fig. 13. Non-haemin iron in bone-marrow and liver in 41 autopsies.

it is usually possible to obtain only specimens of bone-marrow for storage iron estimations it is valuable to study the correlation between bone-marrow iron and liver iron in post-mortem material. As can be seen from Figure 13 those vary more or less independently of each other when the stores of iron are good or abundant; when the concentration of storage iron in the bone-marrow approaches deficiency values, the storage iron of the liver also seems to become scanty (this is the case when the concentration of the non-haemin iron of the bone-marrow falls below 4 mg per 100 g of fresh tissue). When there is a real deficiency of storage iron in the marrow (1–2 mg per 100 g fresh tissue), storage iron is also lacking in the spleen. It is thus necessary only to establish the presence of iron deficiency in the bone-marrow in order to diagnose iron deficiency in the organism. By determining the quantity of non-haemin iron in the bone-marrow fragments obtained on sternal puncture it should be possible to get a good idea of the body's iron stores.

The Non-haemin Iron in Biopsy Specimens of Sternal Marrow

Procedure. Sternal puncture was carried out in the ordinary way. Marrow and blood were drawn into a syringe and transferred to a

Petri dish. Most of the blood was poured off, and the marrow was then washed with physiological saline to remove the blood as completely as possible. The saline was poured off, and the remaining moisture drawn off with a filter paper (Munktell 00). The small fragments of bone-marrow at the bottom of the dish were picked up with a capillary pipette, transferred to a weighing bottle, and weighed. To get reliable figures it is desirable to have at least 50 mg of marrow, but such large quantities are not readily obtainable by sternal puncture.

To the weighed marrow in the weighing bottle was added 2 ml of 5 N HCl, and the specimen placed in a thermostat at 85–90° C. for 1 hour. After cooling, the mixture was transferred to a centrifuge tube. The proteins were precipitated by the addition of 2 ml of 20 % trichloroacetic acid, and the precipitate washed twice with a mixture of equal portions of hydrochloric acid and trichloroacetic acid. The clear supernatants were collected in a 10 ml measuring flask, and one drop of *p*-nitrophenol added. The solution was neutralized with concentrated ammonia to yellow colour. The solution was retitrated with 0.5 N H_2SO_4 until the colour disappeared, when a further 0.3 ml of acid was added and the solution made up to 10 ml with distilled water. To 5 ml of this solution were added 0.05 ml of 2 % hydroquinone and 0.05 ml of 1 % *o*-phenanthroline (test). To the other 5 ml of the solution were added 0.05 ml hydroquinone and 0.05 ml of distilled water (blank). Not less than two hours later the extinction values were read off in a Beckman spectrophotometer, model DU, in 1 cm cuvettes at 510 mμ, the specimen being read against the blank. A further correction was made for the iron content of the chemicals used.

It should be emphasized that the specimens analysed were bone-marrow fragments washed in physiological saline. Determinations of non-haemin iron in rib-marrow from autopsies were done on marrow with its contained blood.

Results and Discussion

The non-haemin iron in bone-marrow fragments obtained on sternal puncture was determined in 12 cases. The results are given in Table 21. The quantity of marrow analysed varied between 22 and 200 mg.

The first five were typical cases of idiopathic hypochromic anaemia, treated as in-patients at Östersund Hospital. (Idiopathic hypochromic anaemia is considerably commoner in this northern district of Sweden than farther south. At the University Hospital, Uppsala, cases of this type are but rarely seen.) In all the cases of idiopathic hypochromic anaemia examined the colour index before treatment was low, varying between 0.43 and 0.63; and the serum iron was low, with values between 4 and 80 μg per 100 ml. The non-haemin iron levels in the sternal marrow were also extremely low, between 1.4 and 1.9 mg per 100 g of tissue.

Case no. 3 received blood transfusions (3 × 400 ml) and 100 mg of iron per day intravenously for ten days. After regenerating blood during 18 days there was still hardly any non-haemin iron to be found in the sternal marrow of this patient; this is probably because most of the iron had been used for synthesis of new haemoglobin. The sixth patient in Table 21 was a 69-year-old woman with pleural empyema and an anaemia with low colour index. The serum iron was low, but this may have been due to the infection. A colour index as low as 0.56, however, is not typical of an anaemia of infection. The sternal marrow in this case contained just as little non-haemin iron as in the five cases of idiopathic hypochromic anaemia, and it may be safely assumed that this patient had, in addition to a pleural empyema, an iron-deficiency anaemia. The seventh patient was a youth of 17 years suffering from ulcerative colitis and chronic hepatitis with intestinal haemorrhage and severe post-haemorrhagic anaemia. The serum iron was low, 45 µg per 100 ml, and the colour index as low as 0.48. In the sternal marrow I found only 1 mg of non-haemin iron per 100 g of fresh tissue.

Compared with the first seven cases the marrow of the remaining five cases had a high concentration of non-haemin iron. Case no. 8, one of Dupley's syndrome, had a low serum iron, 44 µg per 100 ml, but abundant iron in the sternal marrow (29.4 mg per 100 g). It may therefore be suspected that the fall in serum iron was due to infection; Dupley's syndrome in this case may have been due to a rheumatic affection. Case no. 9, with the diagnosis acute leukaemia, had grave anaemia. The high concentration of non-haemin iron in the marrow, 36.2 mg per 100 g, is well in accordance with a fall in total haemoglobin. Case no. 10 had secondary multiple carcinomata of bone, the site of the primary tumour being unknown; here, too, the sternal marrow contained large quantities of non-haemin iron (25.8 mg per 100 g). Case no. 11 was a woman aged 29 with excessive menorrhagia and moderate anaemia; the serum iron was normal and storage iron was still present in the bone-marrow (14 mg of non-haemin iron per 100 g of tissue). The last case was a woman of 52 years with myeloid leukaemia, who had pronounced anaemia with moderate amounts of non-haemin iron in the sternal marrow.

The last five cases bear witness to the reliability of the method described for estimating the non-haemin iron in biopsy specimens of sternal marrow.

It is of great interest that the concentrations of iron which I found in the sternal marrow of idiopathic hypochromic anaemia are as low as the lowest I found in rib-marrow from autopsies. *It has thus been established that in idiopathic hypochromic anaemia there is an absolute deficiency of storage iron in the bone-marrow.* The diminished serum iron in this disease state is thus an expression of a real deficiency of storage iron in the body.

Table 21. Non-haemin iron in sternal marrow (sternal puncture).

Case No., age	Sex	Diagnosis	Hb. conc., g per 100 ml	Red cell count, millions per mm^3	Colour index	Serum iron, µg per 100 ml	Weight of the sample, mg	Non-haemin iron, mg per 100 g fresh tissue
1. M.M., 51	F	Idiopathic hypochromic anaemia	7.3	3.7	0.93	50	100	1.0
2. G.R., 42	F	,, ,, ,,	8.1	4.5	0.90	40	88	1.8
3. S.P., 24	M	(a) ,, ,, ,,	1.8	1.4	0.43	4	106	1.4
		(b) after 1000 mg iron intravenously and 3 × 400 ml blood	8.0	3.0	0.72		94	2.5
4. A.B., 51	F	Idiopathic hypochromic anaemia	7.3	4.0	0.90	64	25	1.5
5. E.E., 40	F	,, ,, ,,	7.0	3.0	0.93	80	22	1.4
6. E.E., 60	F	Rt. pleural empyema + hypochromic anaemia	6.1	3.0	0.50	58	200	1.4
7. N.H., 17	M	[1]Ulcerative colitis + chronic hepatitis	3.8	2.0	0.48	45	75	1.0
8. E.W., 68	M	Dupley's syndrome	12.4	4.2	0.90	44	25	20.4
9. M.W., 75	M	Acute leukaemia	5.8	1.5	1.27	105	50	36.2
10. K.R., 65	M	Secondary carcinoma of bone	12.4	4.2	0.90	123	102	25.8
11. C.H., 29	F	Menorrhagia	10.4	4.0	0.85	85	37	14.0
12. T.P., 52	F	Myeloid leukaemia	7.8	2.7	0.91		22	11.0

[1] Intestinal haemorrhage

In nutritional iron-deficiency anaemia in the rat I found that the plasma iron falls just at the point when the storage iron in bone-marrow, liver, and spleen is exhausted. Iron-deficiency states with low serum iron but no anaemia have been described in man (Jasiński, 1949, 1950; Roth, 1950; and many others). The correlation between serum iron and storage iron in the development of idiopathic hypochromic anaemia in man appears to be similar to that in nutritional iron-deficiency anaemia in the rat.

Concerning the aetiology of idiopathic hypochromic anaemia it may be said, in summary, that in this disease state there is a real deficiency of iron in the body, but that the development of the deficiency is not understood in detail (see also Chapter IV a).

Summary

Before starting the investigations on haemoglobin balance and the storage iron at various levels of iron and protein intake it was first necessary to solve some methodological problems.

The conditions for determining the non-haemin iron in tissue were tested out, and an appropriate procedure established.

A synthetic diet, extremely deficient in iron and producing severe iron deficiency in growing rats, was composed: by using a synthetic diet of this kind the intake of both iron and protein can easily be varied.

It was found that all haemoglobin to within 2–3 % could be removed from the rat body by viviperfusion. The total haemoglobin could thus conveniently be determined with the aid of viviperfusion.

A protein-deficient diet resulted in a slight fall in haemoglobin concentration but a great fall in total haemoglobin. The total haemoglobin diminished more than the carcase protein. Thus, in animals which have not been bled, haemoglobin formation does not have a privileged position over other body proteins in the competition for material for protein synthesis. In adult rats the protein deficiency in the diet began to affect the total haemoglobin at a protein level at which the animals were still able to achieve nitrogen equilibrium and maintain their bodyweight. The fall in total haemoglobin appeared simultaneously with certain other signs of protein deficiency: increased deposition of fat in the liver took place, the percentage content of protein falling; and the kidneys and adrenals diminished in weight in relation to the carcase protein. The percentage composition of the carcase (protein, fat, water, and salts + carbohydrates) remained surprisingly constant even in extreme protein deficiency.

As the total haemoglobin diminished the surplus iron was deposited in the stores.

The low-protein anaemia was microcytic and normochromic or slightly hypochromic. The plasma iron was low during protein deficiency even when the stores contained a surplus of iron.

Pregnancy and lactation were chosen as examples of conditions in which there is heightened competition for protein: in "low-protein animals" the position of haemoglobin formation in this competition was further weakened by pregnancy and lactation, to the extent that when

the protein intake was low enough no haemoglobin at all was formed during the experiment. The haemoglobin concentration gives an incomplete and inaccurate picture of the influence of protein deficiency on haemoglobin formation, especially in such states. For example, in the group with the lowest protein intake, 4.5 % of spray-dried hen's egg-white in the diet, the fall in haemoglobin concentration amounted to about 32 % of the haemoglobin concentration in the controls, whereas the corresponding fall in total haemoglobin was as much as about 58 %.

The supply of storage iron was found to have no influence upon the position of haemoglobin formation in the competition for material for protein synthesis. The deleterious effect of the protein deficiency upon haemoglobin formation was not greater when the supply of storage iron was short, nor did a surplus of storage iron stimulate haemoglobin formation in protein deficiency. When the supply of dietary iron was inadequate the nutritional iron-deficiency anaemia developed in the same way in low-protein animals as in those on an adequate protein intake.

In pure nutritional iron deficiency the haemoglobin concentration fell to the same extent as the total haemoglobin. This applies also to the mean corpuscular haemoglobin, at any rate down to about 40 % of the normal. The mean corpuscular Hb. concentration fell sharply when the anaemia made its appearance, but hardly diminished further as the anaemia progressed.

In nutritional iron deficiency the plasma iron began to show a definite fall just as the last remnants of the storage iron in the liver and spleen were used up. Much of the storage iron of the carcase was still untouched at this stage, and was now drawn upon as the iron-deficiency anaemia progressed. The parenchymatous organs and the carcase were found to contain a fraction of non-haemin iron which could not be utilized for haemoglobin synthesis.

As the protein content of the diet was reduced the weight of the adrenals fell parallel with the total haemoglobin, and a positive correlation was found between total haemoglobin and adrenal-weight (after eliminating the variations in carcase protein). It is recalled that the slight fall in haemoglobin concentration, the low plasma iron, and the fall in total haemoglobin in relation to other body proteins, which were shown to be characteristic of protein deficiency, also occur in adrenal insufficiency. It is suggested that the decrease in total haemoglobin in relation to the carcase protein, and the fall in plasma iron, which occur in protein deficiency, are secondary to adrenal insufficiency, itself caused by the protein deficiency.

When low-protein animals were treated with ACTH or cortisone the carcase protein diminished, the total haemoglobin remaining unchanged. The plasma iron rose, and during cortisone treatment so did the haemoglobin concentration. The increase in plasma iron and haemo-

globin concentration was of the same order as the fall caused by the protein deficiency.

When rats on a low-protein diet were bled they regenerated considerable quantities of haemoglobin, and the protein in the carcase diminished. Increase in adrenal-weight was induced by the bleeding, but it could not be stated whether this was of any significance for the haemoglobin regeneration. The brisk regeneration of blood which follows haemorrhage in rats on a low-protein diet is in accordance with the findings of Whipple *et al.* concerning the high priority of haemoglobin formation in dogs on a protein-deficient diet and subjected to blood-letting.

The non-haemin iron was determined in various human organs, including rib-marrow. The liver, bone-marrow, and spleen may be said to be the most important iron-storage organs of the body, containing as they do the greater part of the storage iron and that which is most readily mobilized at need.

Cases of malignant tumours without haemorrhage often had abundant iron stores. The explanation of this is to be found in the reduction in total haemoglobin which accompanies cachexia and which probably results from the heightened competition for material for protein synthesis. In none of the tumour cases was the increase in storage iron so great that it could not readily be explained by a fall in the total haemoglobin of the order to be expected in cases of malignant tumour.

The iron stores were sometimes large in chronic infections, here again most probably due to a reduction in the total haemoglobin.

It became clear that the quantity of storage iron varies greatly even in "normal" cases: in healthy young men with well-filled iron stores the total amount of storage iron was estimated to be about 1 gram.

The brain contained surprisingly large quantities of non-haemin iron, the amount appearing to be independent of the content of storage iron in the rest of the body.

In cases of cardiac incompensation with pulmonary congestion large quantities of non-haemin iron had been deposited in the lungs.

When the storage iron content of the bone-marrow approached the deficiency level this was also the case in the liver. It is thus possible to diagnose iron deficiency in the organism simply by determining the storage iron in the bone-marrow.

The non-haemin iron was also determined in bone-marrow fragments obtained from twelve patients by sternal puncture. In this way it could be established that there is a complete lack of storage iron in idiopathic hypochromic anaemia.

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